

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**



03-02-04

Image JF 1644

CASE 4-30776A

FILING BY "EXPRESS MAIL" UNDER 37 CFR 1.10

EV335542169US

Express Mail Label Number

March 1, 2004

Date of Deposit

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF

Art Unit: 1644

DIGAN ET AL.

Examiner: Ewoldt, G.

APPLICATION NO: 09/480,236

FILED: JANUARY 10, 2000

FOR: ANTI-CD3 IMMUNOTOXINS AND THERAPEUTIC USES
THEREFOR

MS: Appeal Brief

Commissioner for Patents

PO Box 1450

Alexandria, VA 22313-1450

RESPONSE TO NOTIFICATION OF NON-COMPLIANCE WITH 37 CFR 1.192(c)

Sir:

Appellant's revised Appeal Brief filed on October 15, 2003 has been held to be defective for non-compliance with 37 CFR 1.192(c). Specifically the Brief has been held to not comply for not indicating in what groupings the claims stand or fall and for not providing specific arguments regarding said groupings.

Included herewith is a revised Appeal Brief, in triplicate, indicating that the claims stand or fall together as to each ground of rejection.

Appellant's Appeal Brief is now believed to be in full compliance with 37 CFR 1.192(c).

No fee is believed to be due. If a fee is deemed to be required, the Commissioner is hereby authorized to charge such fee to Deposit Account No. 19-0134.

Respectfully submitted,

Novartis
Corporate Intellectual Property
One Health Plaza, Building 430
East Hanover, NJ 07936-1080
(862) 778-7909

Date: March 1, 2004

Thomas R. Savitsky
Thomas R. Savitsky
Attorney for Applicants
Reg. No. 31,661



FILING BY "EXPRESS MAIL" UNDER 37 CFR 1.10

EV335542169US

Express Mail Label Number

March 1, 2004

Date of Deposit

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF
DIGAN ET AL.

Art Unit: 1644

Examiner: Ewoldt, G.

APPLICATION NO: 09/480,236

FILED: JANUARY 10, 2000

FOR: ANTI-CD3 IMMUNOTOXINS AND THERAPEUTIC USES THEREFOR

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

BRIEF FOR APPELLANTS

Sir:

This is an appeal from the final rejection of Claims 35-54, all of the pending claims in the above-identified application.

I. Real Party In Interest

The real party in interest in the instant appeal is NOVARTIS AG, a company organized under the laws of the Swiss Corporation of 4002 Basle, Switzerland.

II. Related Appeals and Interference

There are no other appeals or interferences known to Appellants, Appellents' legal representative, or assignee which will directly affect or be directly affected by or have a bearing on the Boards' decision in the instant Appeal.

III. Status of the Claims

Claims 1-34 have been cancelled. Claims 35-54 are all of the pending claims in the instant application and such claims were added in an amendment dated August 8, 2002. Claims 35-54 are set forth in attached Appendix A. Claims 35-54 stand rejected and are the claims on Appeal.

IV. Status of Amendments

A "Response to Final Rejection" was submitted on January 31, 2003 but no amendments were entered in said Response. An Advisory Action was mailed on February 24, 2003 in which the Response to Final Rejection was considered. No amendment has been submitted subsequent to the Advisory Action.

V. Summary of the Invention

The present invention concerns a recombinant immunotoxin polypeptide or pharmaceutically acceptable salt thereof containing a CD3-binding domain and a *Pseudomonas* exotoxin (PE) mutant (see, for example, page 6, lines 14-17 of the specification). CD3 is expressed on the surface of T-cells (see, for example, page 1, lines 12-30 of the specification). The immunotoxin of the invention has potent anti-T-cell effects (see, for example, In vivo Applications, page 48, line 1 through page 62, line 7 of the specification and Ex Vivo Applications, page 62, line 9 through page 67, line 6 of the specification). The immunotoxin of the invention is useful for the treatment or prevention of transplant rejection, graft-versus-host disease, T-cell-mediated autoimmune disease, T-cell leukemias or lymphomas which carry the CD3 epitope, acquired immune deficiency syndrome, and other T-cell-mediated diseases and conditions (see, for example, page 6, lines 3-9 of the specification).

VI. Issues

- i) Whether or not the inventors had possession of the subject matter of the invention as claimed in Claims 51-53 at the time the application was filed in order to meet the written description requirement of 35 U.S.C. §112, first paragraph.
- ii) Whether or not Claims 50 and 51 meet the enablement requirement of 35 U.S.C. §112, first paragraph.
- iv) Whether or not Claim 50 contains new matter, i.e., meets the written description requirement of 35 U.S.C. §112, first paragraph.
- v) Whether or not Claims 35-54 are obvious under 35 U.S.C. §103(a) over U.S. Patent No. 6,103,235 ("the '235 patent"), in view of Kreitman et al., *Cancer Biol.*, Vol. 6, pp. 297-306 (1995) ("Kreitman 1995") and Kreitman et al., *Leukemia and Lymphoma*, Vol. 13, pp. 1-10 (1994) ("Kreitman 1994")

VII. Grouping of Claims

The claims stand or fall together as to each ground of rejection.

VIII. Argument

i) Appellants had possession of the subject matter of Claims 51-53 at the time the application was filed and therefore said claims meet the written description requirement of 35 U.S.C. §112, first paragraph.

The Advisory Action refers to the reasons of record set forth regarding amended Claims 31-33 in Paper No. 9. The relevant portion of Paper No. 9 recites:

...the specification provides an insufficient written description of antibodies having a variable region which is at least 99% identical to the variable region of UCHT-1 and is at least 95% as effective on a molar basis in competing with UCHT-1. No such variants of the UCHT-1 antibody are disclosed in the specification. Given the essentially unlimited number of antibodies encoded by a virtually unlimited number of polynucleotides encompassed by the claims, one of skill in the art would conclude that the specification fails to disclose a representative number of species to describe the claimed genus. See *Eli Lilly*, 119 F.3d 1559, 43 USPQ2d 1398.

Appellants wish to point out that Claim 51 is somewhat different from cancelled Claim 33 which was subject to the above reasons for rejection. Claim 51 requires an antibody having a variable region which is at least 90% identical to the variable region of UCHT-1 as determined by use of the Bestfit program. The version of cancelled Claim 33 that was subject to the above reasons for rejection recited "99% identical" and did not refer to the Bestfit program. Also, Claim 51 requires that the antibody "is at least 90% as effective on a molar basis in competing with UCHT-1 for binding to human CD3 antigen and having at least one sequence segment of at least five amino acids of human origin". The version of cancelled Claim 33 that was subject to the above reasons for rejection recited "95% as effective...". Nevertheless the reasoning for the rejection appears to be the same regardless of whether 90 or 99 % identity is recited and whether 90 or 95% effectiveness is recited.

The variable region of antibody UCHT-1 is taught in Appellants' specification to be of a specific amino acid sequence, i.e., comprising residues 3 to 112 (light chain) and 128 to 249 (heavy chain) of SEQ. ID. NO.:1. The Examiner states in the Final Rejection (paper 22) at the bottom of page 2 that:

It remains the Examiner's position that this disclosure, while describing SEQ.ID. NO.:1, provides an inadequate description of sequences that are at least 90% identical to the variable region of

UCHT-1 or antibodies about 90% as effective as UCHT-1 for binding human CD3.

As understood by Appellants, there are two aspects to the present ground of rejection; the first concerns the 90% identity language and the second concerns the 90% effectiveness language.

The Board's attention is directed to "Written Description" requirement, 66 Fed. Reg. 1099 (January 5, 2001) incorporated into the MPEP as Section 2163 ("Guidelines"); Synopsis of Application of Written Description ("Application of Guidelines"), available at <http://www.uspto.gov/web/patents/guides.htm>; and Enzo Biochem Inc. v. Gen-Probe Inc., 63 USPQ2nd 1609 (Fed. Cir., 2002).

In the Enzo case, the CAFC cites the Guidelines and adopts the PTO's standards for determining compliance with the written description requirement where a functional characteristic is coupled with a disclosed correlation between that function and a structure that is sufficiently known or disclosed (Enzo at 1613). It is Appellants' position that the Enzo case is controlling for the present situation. The Examiner has acknowledged that the specification teaches a specific sequence (a "structure"). Appellants' Claim 51 effectively recites this structure as acknowledged by the Examiner. Appellants' Claim 51 further recites a functional characteristic, i.e., the 90% effectiveness language. Appellants' Claim 51 further recites a specific correlation between the structure and the functional characteristic, i.e., the 90% identity language. Another requirement is present in Claim 51, i.e., that at least one sequence segment is of at least five amino acids of human origin, but this aspect does not appear to be germane to the rejection. Thus, all requirements enunciated by the CAFC in the Enzo case are present in Appellants' Claim 51. Further in Enzo at 1615, the CAFC points to Example 9 of the Application of Guidelines with favor. In the analysis of Example 9 of the Application of Guidelines, it is stated on page 36:

A review of the full content of the specification indicates that the essential feature of the claimed invention is the isolated nucleic acid that hybridizes to SEQ ID NO: 1 under highly stringent conditions and encodes a protein with a specific function. The art indicates that hybridization techniques using a known DNA as a probe under highly stringent conditions were conventional in the art at the time of filing.

The claim is drawn to a genus to nucleic acids all of which must hybridize with SEQ ID NO: 1 and must encode a protein with a specific activity. ...

There is a single species disclosed (a molecule consisting of SEQ ID NO: 1) that is within the scope of the claimed genus.

There is actual reduction to practice of the disclosed species.

Now turning to the genus analysis, a person of skill in the art would not expect substantial variation among species encompassed within the scope of the claims because the highly stringent hybridization conditions set forth in the claim yield structurally similar DNAs. Thus, a representative number of species is disclosed, since highly stringent hybridization conditions in combination with the coding function of DNA and the level of skill and knowledge in the art are adequate to determine that applicant was in possession of the claim invention.

Conclusion: The claimed invention is adequately described.

The facts of the present application are analogous to Example 9 of the Application of Guidelines. Appellants' Claim 51 provides a specific sequence (species) coupled with a functional limitation to describe a particular genus and a correlation between the two. Therefore, Appellants' specification provides a sufficient written description of the invention as defined in Claim 51 (and Claims 52 and 53 dependent thereon) to comply with the requirements of 35 U.S.C. §112, first paragraph.

The Examiner cites Eli Lilly, 119 F.3d 1559, 43 USPQ2d 1398 to support this ground for rejection. However, as the CAFC points out in Amgen Inc. v. Hoeschst Marion Roussel Inc., 65 USPQ2d, 1385 (Fed. Cir 2003):

More recently, in Enzo Biochem, we clarified that Eli Lilly did not hold that all function descriptions of genetic material necessarily fail as a matter of law to meet the written description requirement; rather, the requirement may be satisfied if in the knowledge of the art the disclosed function is sufficiently correlated to a particular, known structure. See Enzo Biochem, 296F.3d at 1324, 63 USPQ2d at 1613.

In the Advisory Action the Examiner holds that Enzo is not applicable because Enzo concerned hybridization under stringent conditions whereas Claims 51-53 recite 90% identity. It is Appellants' position that in the context of Enzo and Claims 51-53, 90% identity is analogous to hybridization under stringent conditions. The CAFC did not hold that only hybridization under stringent conditions can meet the written description requirement. For example, the CAFC in Enzo indicates that a claim such as "an isolated antibody capable of binding to antigen X" will meet the written description requirement, "notwithstanding the functional definition of the antibody in light of the well-defined structural characteristics ... of antibody" (Enzo at 1613). Appellant's Claims 51-53 have well-defined structural characteristics, i.e., an antibody having a variable region of antibody UCHT-1, and at least 90% identity to said variable region of UCHT-1.

Furthermore, Appellants' specification teaches a specific method for determining sequence identity. The last paragraph of page 22 of the specification states:

As a practical matter, whether any particular polypeptide sequence is at least 80%, 90%, or at least 95%, "identical to" another polypeptide can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711). When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% or the total number of amino acid residues in the reference sequence are allowed.

Appellant's Claim 51 specifically recites that the percent identity is determined by use of the Bestfit program. Thus, one skilled in the art will have no trouble determining whether or not a particular sequence will meet the 90% identity requirement of Appellants' Claims 51-53.

Regarding the determination of antibodies that are about 90% as effective as UCHT-1 for binding human CD3, Appellant's specification on page 21 states:

These antibodies include a monoclonal antibody competing with, e.g., UCHT-1, for binding to human CD3 antigen at least about 80%, and more preferably at least about 90%, as effectively on a molar basis as UCHT-1, and having at least one sequence segment of at least five amino acids of human origin. By "specific binding affinity" is meant binding affinity determined by noncovalent interactions such as hydrophobic bonds, salt linkages, and hydrogen bonds on the surface of binding molecules.

Moreover, determining the binding affinity of a given antibody for CD3 relative to UCHT-1 is well within the skill in the art, see, e.g., the competitive FACS assay for binding in the article by J.M. Hexham et al., *Mol. Immunol.*, Vol. 38, pp. 397-408 (2001), included herewith as Attachment A. From the information available in the art and the teachings in the specification, it is submitted that one skilled in the art would have no problem determining those monoclonal antibodies that are about 90% as effective as UCHT-1 for binding human CD3.

ii) Claims 50 and 51 meet the enablement requirement of 35 U.S.C. §112, first paragraph.

In the Advisory Action it is stated:

Claims 50-51 stand rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for

a recombinant immunotoxin polypeptide consisting of the polypeptide encoded by the nucleotide sequence of SEQ ID NO:2, does not reasonably provide enablement for:

a recombinant immunotoxin polypeptide comprising an antibody having a variable region which is at least about 90% identical to the variable region of UCHT-1 and is at least about 90% as effective as UCHT-1 for binding human CD3, for the reasons of record as set forth in the rejections of Claims 31-33 in Paper No. 17, mailed 3/28/02.

The reasons for the rejection in Paper No. 17 are stated as:

Claims 31-33 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for, a recombinant immunotoxin polypeptide consisting of the polypeptide encoded by the nucleotide sequence of SEQ ID NO:2, does not reasonably provide enablement for:

- A) a recombinant immunotoxin polypeptide comprising the polypeptide encoded by a nucleotide sequence which hybridizes with the nucleotide sequence of SEQ ID NO:2 under stringent hybridization conditions, or
- B) a recombinant immunotoxin polypeptide comprising the polypeptide encoded by any nucleotide sequence which hybridizes to the nucleotide sequence of Claim 31 under stringent hybridization conditions, or
- C) a recombinant immunotoxin polypeptide comprising an antibody having a variable region which is at least about 90% identical to the variable region of UCHT-1 and is at least about 90% as effective as UCHT-1 for binding human CD3.

Reasons A) and B) as recited in Paper No. 17 are not referred to in the Advisory Action, therefore it is not clear if these reasons for rejection have been overcome. An amendment to Claim 50 was made subsequent to Paper No. 17 which is believed to have overcome Reasons A) and B) (this amendment prompted issue iv) which will be addressed *supra*). In the event Reasons A) and B) have not been overcome, then Appellants will address these reasons for rejection in a Reply Brief.

It is further stated in Paper No. 17:

The specification disclosure is insufficient to enable one skilled in the art to practice the invention as claimed without an undue amount of experimentation. Undue experimentation must be considered in light of factors including: the breadth of the claims, the nature of the invention, the state of the prior art, the level of one of ordinary skill in the art, the level of predictability of the art, the amount of direction provided by the inventor, the existence of working examples, and the quantity of experimentation needed to make or use the invention....

Regarding C), it is well-established that changes in the amino acid sequence of the variable region of an antibody create new antibodies with highly unpredictable binding characteristics. See, e.g., Kussie et al. (1995), Table I, which teaches that the substitution of a single amino acid can totally ablate antigen binding. As a further demonstration of the unpredictability of substituted or mutated antibodies, see Chen et al. (1995). The reference again teaches that the substitution of a single amino acid can totally ablate antigen binding (Figure 1), however, the reference additionally teaches that the same substitution in closely related antibodies can have opposite effects. The authors compared the effects of identical substitutions in related antibodies D16 and T15, and as shown in Figure 3, some substitutions increased antigen binding in one antibody while ablating it in the other. The reference serves to demonstrate the highly unpredictable nature of substituted antibodies and thus, the highly unpredictable nature of the antibody of the instant claims. Given said unpredictability, significant direction would be required to make and use the instant invention as claimed. However, the specification discloses just a single recombinant immunotoxin antibody (other than that encoded SEQ ID NO:2) with a single mutation in a residue outside the CDR antigen binding domains. Said disclosure is insufficient to enable the unlimited number of immunotoxins encompassed by Claim 33.

In re Wands, 858 F.2d at 737, 8 USPQ2d at 1404 (Fed. Cir. 1988) indicates that the more unpredictable an area is, the more specific enablement is necessary in order to satisfy the statute. In view of the quantity of experimentation necessary, the lack of sufficient working examples encompassing the entirety of the claimed methods, the unpredictability of the art, and the breadth of the claims, it would take undue trials and errors to practice the claimed invention.

While Appellants agree that small changes in an antibody sequence can have a dramatic effect on activity, Appellants have provided sufficient information to enable one skill in the art to practice the invention as claimed.

It is submitted that the scope and detail of Appellants' disclosure do fairly provide suitable procedures for obtaining the immunotoxins of the invention. It is respectfully submitted that if the work described in the specification may be labor-intensive and time-consuming, it does not negative Appellants' compliance with the enablement requirement (see Hybritech, Inc. v. Monoclonal Antibodies, Inc., 802 F.2d 1367 (Fed. Cir. 1986), *cert. denied*, 480 U.S. 947 (1987) and *In re Wands*, 858 F.2d 731, 8 USPQ2d 1400 (Fed. Cir. 1988)).

Similarly, while possibly labor-intensive or time-consuming, it would be well within the range of normal experimentation by a worker of ordinary skill in the art, using the procedures disclosed by Appellants to prepare immunotoxins not exemplified in the specification. The skill in the art at the time of filing the application was very high. One skilled in the art could easily determine whether or not a particular polypeptide has 90% identity to the variable region of

UCHT-1 and whether such a polypeptide is at least about 90% as effective as UCHT-1 for binding human CD3.

Regarding the "90% identity" to UCHT-1 requirement, the specification teaches the use of the Bestfit program as described above in Argument i).

Regarding the determination of antibodies that are about 90% as effective as UCHT-1 for binding human CD3, the Board's attention is directed to the information described above in Argument i).

iv) Claim 50 does not contain new matter and therefore meets the written description requirement of 35 U.S.C. §112, first paragraph.

It is stated in the Advisory Action that:

Claim 50 stands rejected under 35 U.S.C. §112, first paragraph, as the specification does not contain a written description of the claimed invention, in that the disclosure does not reasonably convey to one skilled in the relevant art that the application was filed for the reasons of record as set forth in Paper No. 22, mailed 11/21/02. This is a new matter rejection.

Regarding the rejection of Claim 50, Paper No. 22 states:

The specification and the claims as originally filed do not provide support for the invention as now claimed, specifically, "the polypeptide encoded by the complement of a nucleotide sequence having at least 300 bases which hybridizes to the nucleotide sequence of Claim 49 (SEQ. ID. NO.2)."

Applicant's amendment, filed 8/08/02, asserts that no new matter has been added. Applicant has indicated that at page 38 support for the new limitation can be found. However, no support for the complement of a polynucleotide having at least 300 bases has been found on the page.

The test for sufficiency of support is whether the disclosure of the application reasonably conveys to the skilled artisan that the inventor had possession of the claimed subject matter. However, it is well-settled that it is not necessary that the claimed subject matter be described in *ipsis verbis* to satisfy the written description requirement of 35 U.S.C. §112 (see, e.g., Purdue Pharma L.P. v. Faulding Inc., 56 USPQ2d 1481 (Fed. Cir. 2000)). Furthermore, explicit support is not required, inherent support is sufficient (see, e.g., Standard Oil Co. v. Montedison. S.p.A., 206 USPQ 676 (D. Del. 1980), *aff'd*, 212 USPQ 327 (3d Cir. 1981), *cert. denied*, 456 U.S. 915 (1982)).

On Page 38 of the specification, the last clause of the second paragraph states: "... as well as complementary strands of the foregoing nucleic acids".

The third paragraph on Page 38 describes the polynucleotides of at least 300 bases "which hybridizes to a polynucleotide which encodes a polypeptide of the invention".

Clearly, from Appellant's specification and the relationship between polynucleotides and their complements as is well-known in the art, Appellants were in possession of the subject matter of Claim 50 upon filing of the application.

v) Claims 35-54 are patentable under 35 U.S.C. §103(a) over the '235 patent in view of Kreitman 1995 and Kreitman 1994.

It is stated in the Advisory Action that:

Claims 35-54 are rejected under 35 U.S.C. §103(a) as being unpatentable over U.S. Patent No. 6,103,235 (2000, of record) in view of Kreitman et al. (1995, of record) and Kreitman et al. (1994, of record), for the reasons of record as set forth in the rejections of Claims 1-7, 9-16, 29-30 and 33-34, in Paper No. 17, mailed 3/28/02.

The reasons for the above-referenced rejection in Paper No. 17 is as follows:

The '235 patent teaches a recombinant immunotoxin polypeptide (RIP) comprising a single-chain Fv (which is an F_{ab} fragment) anti-human UCHT-1 CD3 ϵ binding domain and a diphtheria toxin (DT) (an ADP-ribosylating exotoxin) (see entire document, particularly, column 19, lines 21-30). The reference further discloses a RIP pharmaceutical composition comprising a single-chain Fv fused to the carboxy terminus of the exotoxin in a V_L - L - V_H - C - exotoxin conformation (see particularly Figure 12).

The reference teachings differ from the claimed invention in that they do not teach the use of PE38 as the ADP-ribosylating exotoxin in the RIP construct.

Kreitman et al. (1995) teaches immunotoxic antibody-PE38 fusion proteins and antibody-PE40 fusion proteins (see particularly, Figure 1). The reference further teaches that the PE38 and PE40 immunotoxins are functionally interchangeable (see particularly Figure 2A).

Kreitman et al. (1994) teaches immunotoxic antibody-PE40 fusion proteins and immunotoxic antibody-DT fusion proteins, and that they are functionally interchangeable (see particularly, Figure 2 and Table 2).

From the teachings of the references it would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to produce a pharmaceutical composition

comprising a RIP comprising a single-chain Fv (which is an F_{ab} fragment) anti-human UCHT-1 CD3 $\epsilon\gamma$ binding domain, further comprising a single-chain Fv fused to the carboxy terminus of the exotoxin in a V_L - L - V_H - C - exotoxin conformation (the polypeptide of SEQ ID NO:1, encoded by the nucleotide of SEQ ID NO:2), as taught by the '235 patent substituting the PE38 exotoxin for the DT exotoxin, as taught by Kreitman et al. (1994 and 1995). One of ordinary skill in the art would have been motivated to make said substitution because PE38 exotoxin was a well-known equivalent for PE40 exotoxin which was a well-known equivalent for the DT exotoxin disclosed in the '235 patent" as demonstrated by Kreitman et al. (1995 and 1994). The substitution of known equivalents is considered obvious (see MPEP 2164.06) and one of ordinary skill in the art would have a reasonable expectation of success in making said substitution. Note that the claim limitation of a PE mutant having ADP-ribosylating and translocation functions but substantially-diminished cell-binding ability recited in Claim 1 merely comprises a functional characteristic of the PE38 mutant taught by Kreitman et al. (1995). Likewise, the claim limitations of Claims 3-11, regarding the CD3-binding domain, e.g., an anti-CD3-binding fragment which binds an epitope on the CD3 chain comprising a single-chain Fv, are functional characteristics of the UCHT-1 antibody and thus characteristics of the UCHT-1 construct of the '235 patent.

Although the bits and pieces of Appellants' claimed immunotoxin may be present in the prior art, the requisite incentive or motivation to combine these bits and pieces is lacking.

It is stated in Paper No. 17 that Kreitman (1994) teaches that immunotoxic antibody-PE40 fusion proteins and immunotoxic antibody-DT fusion proteins are functionally interchangeable. It is respectfully pointed out that this reference does not teach such interchangeability.

Kreitman 1994 compares the activities of several immunotoxins directed against Tac (not CD3 as presently claimed). The data in Tables 3 and 4 of the reference show the ability of different patients blood cells to be killed by the different immunotoxins. There is no predictability or pattern to the results. That is, *a priori*, one could not predict the effectiveness of a given PE-Tac immunotoxin on a given cell population by knowing the activity of a given DT-Tac immunotoxin on that cell population.

Moreover, attention is directed to the reference by Batra et al. *Mol. Cell. Biol.*, Vol. 11, No. 4, pp. 2200-2205 (1991) (included herewith as Attachment B). this reference compares two single-chain anti-human transferrin receptor immunotoxins, one with DT (designated DT388-anti-TFR (Fv)) and one with PE (designated TFR (Fv) - PE40). In the Discussion section, it is concluded that:

Unexpectedly, large differences in the activities of the two single-chain immunotoxins were observed. On some cell lines (A431,

KB and MCF7) anti-TFR (Fv) - PE40 was at least 100-fold more active than DT-388-anti-TFR (Fv). On two cell lines (HUT102 and HT29), DT388-anti-TFR (Fv), was about three-fold more active.

Thus, one skilled in the art having the prior art before him could not *a priori* reasonably predict the effectiveness for a particular use of an anti-CD3-PE based immunotoxin with the knowledge that an anti-CD3-DT based immunotoxin is effective for that use.

Regarding Appellant's above arguments, it is stated in the Final Rejection:

It is the Examiner's position that true or not, Applicant's assertions are irrelevant. The instant claims are drawn to a product, i.e., an immunotoxin, not a method of using an immunotoxin. Accordingly, the references need only provide a motivation to make the immunotoxin of the instant claims. While it may be that the DT versus PE immunotoxins vary in cytotoxicity, by an order of magnitude in some instances, they all remain cytotoxic. Thus, in at least that aspect they are interchangeable. Applicant also asserts that the asserted variable cytotoxicity is unpredictable, if that is so, then the skilled artisan would have the additional motivation of preparing the immunotoxin of the instant claims to see if it possessed the sought after degree of cytotoxicity. Contrary to Applicant's assertion, predictability for any particular use need not be the motivation for preparing the product of the instant claims

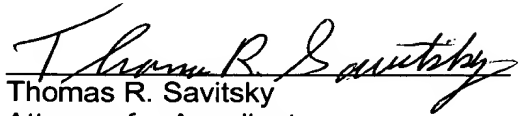
The Examiner contends that because Appellants are claiming a product, not a method, the above arguments are irrelevant. It is respectfully pointed out that this is manifestly not the case in U.S. patent law. As far back as the seminal case of In re Papesch, 137 USPQ 43 (CCPA 1963), the courts have recognized that it is an error of law to fail to take into consideration the biological or pharmaceutical property of a claimed composition of matter.

While assuming, *arguendo*, that Appellants' claimed invention may be "obvious to try" or "obvious to experiment", it is well-established that these are not the standards for obviousness under 35 U.S.C. §103 (see, e.g., In re Mercier, 185 USPQ 774 (CCPA 1975) and In re Dow Chemical Co., 5 USPQ2d 1529 (Fed. Cir. 1988)). The test for obviousness is not whether or not it would have been obvious to test Appellants' immunotoxins; but rather, whether or not it would be obvious that Appellants' immunotoxins would be successful. Because of the variability of immunotoxins as demonstrated in the cited art, it would not be obvious that Appellants' invention would be successful.

For the above-stated reasons Appellants respectfully request allowance of Claims 35-54 in the application.

Respectfully submitted,

Novartis
Corporate Intellectual Property
One Health Plaza, Building 430/2
East Hanover, NJ 07936-1080


Thomas R. Savitsky
Attorney for Appellants
Reg. No. 31,661
(862) 778-7909

TRS/ld

Encl.: Appendix - The Claims in Appeal
Attachment A - J.M. Hexham et al., *Mol. Immunol.*, Vol. 38, pp. 397-408 (2001)
Attachment B - Batra et al. *Mol. Cell. Biol.*, Vol. 11, No. 4, pp. 2200-2205 (1991)

Date: March 1, 2004

Appendix

35. A recombinant immunotoxin polypeptide or a pharmaceutically acceptable salt thereof comprising a CD3-binding domain and a *Pseudomonas* exotoxin (PE) mutant, said PE mutant having ADP-ribosylating and translocation functions but substantially diminished cell-binding ability.
36. A recombinant immunotoxin polypeptide or a pharmaceutically acceptable salt thereof according to claim 35 wherein the CD3-binding domain comprises an anti-CD3 antibody or CD3-binding fragment thereof.
37. A recombinant immunotoxin polypeptide or a pharmaceutically acceptable salt thereof according to claim 36 wherein the anti-CD3 antibody or CD3-binding fragment thereof binds an epitope on the ϵ chain of human CD3.
38. A recombinant immunotoxin polypeptide or a pharmaceutically acceptable salt thereof according to claim 36 wherein the anti-CD3 antibody or CD3-binding fragment thereof binds an epitope formed by the ϵ and γ chains of human CD3.
39. A recombinant immunotoxin polypeptide or a pharmaceutically acceptable salt thereof according to claim 36 wherein the CD3-binding domain comprises a Fab fragment of an anti-CD3 antibody.
40. A recombinant immunotoxin polypeptide or a pharmaceutically acceptable salt thereof according to claim 36 wherein the CD3-binding domain comprises the Fv region, or a CD3-binding fragment thereof, of an anti-CD3 antibody.
41. A recombinant immunotoxin polypeptide or a pharmaceutically acceptable salt thereof according to claim 36 wherein the CD3-binding domain comprises monoclonal antibody UCHT-1 or a CD3-binding fragment thereof.
42. A recombinant immunotoxin polypeptide or a pharmaceutically acceptable salt thereof according to claim 36 wherein the CD3-binding domain comprises a single chain Fv of an anti-CD3 antibody.
43. A recombinant immunotoxin polypeptide or a pharmaceutically acceptable salt thereof according to claim 35 comprising a single chain Fv of UCHT-1 fused to a PE mutant essentially deleted of its cell-binding domain.
44. A recombinant immunotoxin polypeptide or a pharmaceutically acceptable salt thereof according to claim 43 wherein the PE mutant is PE38.

45. A recombinant immunotoxin polypeptide or a pharmaceutically acceptable salt thereof according to claim 43 consisting essentially of the single chain Fv of an anti-human CD3 antibody fused via the carboxy terminus thereof to a PE mutant essentially deleted of its cell-binding domain.
46. A recombinant immunotoxin polypeptide or a pharmaceutically acceptable salt thereof according to claim 45 having the formula $V_L - L - V_H - C - PE$ mutant.
47. A recombinant immunotoxin polypeptide or a pharmaceutically acceptable salt thereof according to claim 46 wherein V_L and V_H are derived from UCHT-1 and the PE mutant is PE38.
48. A pharmaceutical composition comprising a recombinant immunotoxin polypeptide or a pharmaceutically acceptable salt thereof according to claim 35 in a pharmaceutically acceptable carrier.
49. A recombinant immunotoxin polypeptide or a pharmaceutically acceptable salt thereof, wherein the polypeptide comprises the polypeptide coded for by the nucleotide sequence shown in Figure 15 (SEQ. ID. NO:2).
50. A recombinant immunotoxin polypeptide or a pharmaceutically acceptable salt thereof, wherein the polypeptide comprises the polypeptide encoded by the complement of a nucleotide sequence having at least 300 bases which hybridizes to the nucleotide sequence of claim 49 (SEQ. ID. NO:2) under stringent hybridization conditions.
51. A recombinant immunotoxin polypeptide or a pharmaceutically acceptable salt thereof according to claim 36, wherein the CD3-binding domain comprises the Fv region, or a CD3-binding fragment thereof of an antibody selected from: monoclonal antibody UCHT-1, an antibody having a variable region which is at least 90% identical to the variable region of UCHT-1 as determined by use of the Bestfit program and is at least about 90% as effective on a molar basis in competing with UCHT-1 for binding to human CD3 antigen and having at least one sequence segment of at least five amino acids of human origin.
52. A recombinant immunotoxin polypeptide or a pharmaceutically acceptable salt thereof according to claim 51, wherein the Fv region is a single-chain Fv.
53. A recombinant immunotoxin polypeptide or a pharmaceutically acceptable salt thereof according to claim 52, wherein the CD3-binding fragment comprises a single-chain Fv of UCHT-1.
54. A recombinant immunotoxin polypeptide selected from polypeptides having residues 1-601, 2-601 or 3-601 of SEQ. ID. NO:1 or a pharmaceutically acceptable salt thereof.



Influence of relative binding affinity on efficacy in a panel of anti-CD3 scFv immunotoxins

J. Mark Hexham^{a,*}, Debra Dudas^a, Ron Hugo^a, Jerry Thompson^b, Vicki King^a, Carol Dowling^a, David M. Neville Jr^c, Mary Ellen Digan^a, Phil Lake^a

^a Transplantation Research, Novartis Pharmaceuticals, 556 Morris Avenue, Summit, NJ 07901, USA

^b Fenske Laboratory, Pennsylvania State University, University Park, PA 16802, USA

^c Laboratory of Molecular Biology, Section on Biophysical Chemistry, National Institute of Mental Health, Bethesda, MD 28092, USA

Received 4 June 2001; received in revised form 13 August 2001; accepted 16 August 2001

Abstract

The in vitro cell killing potency of an immunotoxin reflects the aggregate of several independent biochemical properties. These include antigen binding affinity; internalization rate, intracellular processing and intrinsic toxin domain potency. This study examines the influence of antigen binding affinity on potency in various immunotoxin fusion proteins where target antigen binding is mediated by single chain antibody variable region fragments (scFv). Firstly, the relationship between affinity and potency was examined in a panel of four scFv immunotoxins generated from different anti-CD3 monoclonal antibodies fused to the 38 kDa fragment of *Pseudomonas aeruginosa* exotoxin A (PE38). Of these four scFv-PE38 immunotoxins, the one derived from the anti-CD3 monoclonal antibody UCHT1 has highest cell killing potency. Analysis of these four scFv-PE38 immunotoxins indicated a correlation between antigen binding affinity and immunotoxin potency in the cell killing assay with the exception of the scFvPE38 immunotoxin derived from the antibody BC3. However this scFv appeared to suffer a greater drop in affinity ($\sim 100\times$), relative to the parent Mab than did the other three scFvs used in this study ($2\text{--}10\times$). Secondly, the scFv(UCHT1)-PE38 immunotoxin was then compared with a further panel of scFv(UCHT1)-derived immunotoxins including a divalent PE38 version and both monovalent and divalent *Corynebacterium diphtheriae* toxin (DT389) fusion proteins. When the scFv-UCHT1 domain was amino-terminally positioned relative to the toxin, as in the scFv(UCHT1)-PE38, an approximately 10-fold higher antigen-binding affinity was observed than with the C-terminal fusion, used in the DT389-scFv(UCHT1) molecule. Despite this lower antigen-binding activity, the DT389-scFv immunotoxin had a 60-fold higher potency in the T-cell-killing assay. Thirdly, a divalent form of the DT389-scFv construct, containing tandem scFv domains, had a 10-fold higher binding activity, which was exactly reflected in a 10-fold increase in potency. Therefore, when comparing immunotoxins in which scFvs from different antibodies are fused to the same toxin domain (DT or PE) a broad correlation appears to exist between binding affinity and immunotoxin potency. However, no correlation between affinity and potency appears to exist when different toxin domains are combined with the same scFv antibody domain. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Immunotoxin; Affinity; CD3; Transplantation; Tolerance; Antibody engineering

1. Introduction

Immunotoxins have been used as both chemical conjugates and single-chain antibody fusion proteins in several clinical situations, especially as anti-tumor agents (reviewed by Frankel et al., 1995; Pai and Pastan, 1998). While a wide variety of toxins including

ricin, saporin and abrin have been used in clinical trials, recombinant immunotoxins derived from *Pseudomonas aeruginosa* (exotoxin A, Pai and Pastan, 1998) and *Corynebacterium diphtheriae* (Diphtheria toxin, Foss et al., 1998) have shown most potential for clinical development. A diphtheria toxin-based fusion molecule DAB₃₈₉IL-2 has recently been approved for systemic treatment of cutaneous T cell lymphoma following demonstration of efficacy in clinical trials (Saleh et al., 1998; Duvic et al., 1998; LeMaistre et al., 1998).

* Corresponding author. Fax: +1-908-277-5035.

E-mail address: mark.hexham@pharma.novartis.com (J.M. Hexham).

CD3 is a polypeptide complex associated with the T-cell receptor on the surface of all peripheral T-cells and mature thymocytes (Clevers et al., 1988), making it an attractive target for an immunosuppressive immunotoxin. A chemical conjugate of an anti-primate CD3 ϵ antibody (FN18, Nooij et al., 1986) with a cell-binding domain mutated form of diphtheria toxin (CRM9) has been used in primates to generate long-term graft survival without chronic immunosuppression in kidney allotransplantation (Knechtle et al., 1997; Thomas et al., 1997; Contreras et al., 1998), concordant xenogeneic islet transplantation (Thomas et al., 1999) and islet allotransplantation (Thomas et al., 2000). These studies set a precedent for use of anti-CD3 immunotoxins in solid organ transplantation. The production of a clinically useful product for such a protocol would be desirable, i.e. a human T-cell-specific immunotoxin as a scaleable recombinant fusion protein. In the present study several anti-CD3 immunotoxins (Fig. 1) were produced and evaluated for relative CD3 binding affinity and efficacy in T-cell killing, with a view to their use in tolerance induction for solid organ transplantation. Overall, there was a broad correlation between affinity and potency, for different immunotoxins based on a particular toxin domain (either DT or PE). However, no correlation was observed between affinity and efficacy when comparing fusion proteins comprising different toxin domains in combination with the same single chain antibody variable region fragments (scFv) domain.

2. Materials and methods

2.1. General materials and methods

Plasmid DNA encoding full-length diphtheria toxin was prepared from ATCC culture #67011: JM109 cells carrying insert PD γ 2 in vector PEMBL8+. BL21 cells and pET15b vector were from Novagen. Chemicals were from Sigma; molecular biology enzymes were from Gibco-BRL; *Pfu* polymerase was from Stratagene, AmpliTaq was from Perkin-Elmer. PCR amplification reactions were carried out on a Perkin-Elmer 9600 thermal cycler, and molecular biology procedures were carried out according to Sambrook et al. (1989). DNA sequencing was performed on an Applied Biosystems 373A automated sequencer according to manufacturer's protocol. Where necessary correction of DNA sequences was carried out using appropriately designed mutagenic primers and the QuikChange kit (Stratagene).

2.2. Cloning, expression and purification of immunotoxins

The SP34 and BC3 antibodies were purified from hybridoma supernatants by Protein A affinity chromatography. The heavy and light chains were separated by SDS-PAGE under reducing conditions, transferred to PVDF membrane and subjected to N-terminal sequencing on a HP G1000A protein sequencer

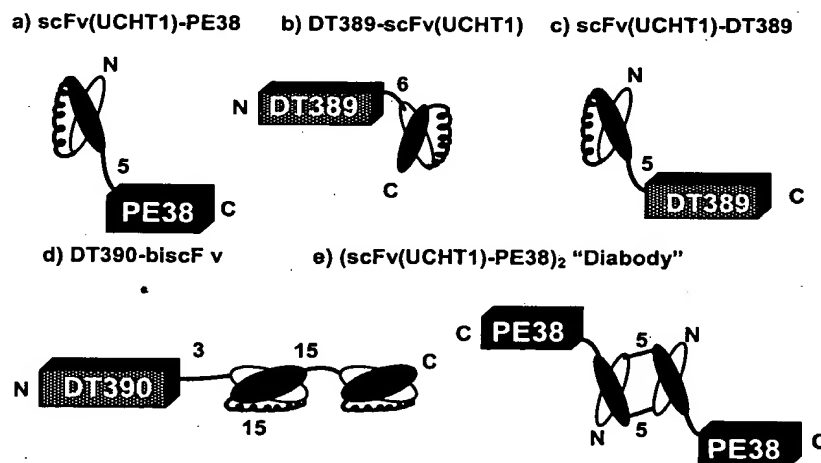


Fig. 1. Design of UCHT1-based scFv immunotoxins used in this study. (a) scFv(UCHT1)-PE38, a monovalent scFv-Pseudomonas exotoxin A fusion. (b) DT389-scFv(UCHT1), a monovalent diphtheria toxin-scFv fusion. (c) scFv(UCHT1)-DT389 (DT389-scFv(UCHT1) Reverse) a construct with the two domains from DT389-scFv(UCHT1) in the reverse orientation. (d) DT390-biscFv(UCHT1), a divalent DT390 fusion to tandem C-terminal scFv domains. (e) Diabody [scFv(UCHT1)-PE38]₂, is a divalent scFv-Pseudomonas exotoxin A fusion protein. N and C denote amino and carboxy termini, respectively. Numbers denote lengths of inter-domain linkers in amino acid residues. Open and filled ovals represent VL and VH domains, respectively.

Table 1

Characteristics of anti-CD3 monoclonal antibodies from which scFv based immunotoxins were generated in this study

Antibody	Specificity	Species	Subclass	Reference
UCHT1	Human CD3 ϵ	Mouse	IgG1/ κ	Beverley and Callard, 1981
SP34	Human and Primate CD3 ϵ	Mouse	IgG3/ λ	Sancho et al., 1992
BC3	Human CD3 ϵ	Mouse	IgG2b/ κ	Anasetti et al., 1992
145-2C11	Mouse CD3 ϵ	Hamster	IgGgr1/ κ	Leo et al., 1987

(Hewlett–Packard), as described by Matsudaira (1987). The N-terminal protein sequence was used to design appropriate 5' primers to amplify SP34 and BC3 VH and VL genes. 3' primers for VH and VL genes were designed based on mouse J segment consensus sequences. Genes encoding scFv were assembled by PCR to splice the VL and VH encoding segments with a flexible linker encoding segment (Huston et al., 1988). scFv fragments encoding UCHT1 and 145-2C11 were constructed based on published sequence data (Shalaby et al., 1992; Gilliland et al., 1996, respectively).

scFv-PE38 immunotoxin gene fusions (e.g. Fig. 1a) were produced from UCHT1, BC3, SP34 and 145-2C11 (Table 1) scFvs by fusing them 5' to the PE38 encoding gene fragment of *P. aeruginosa* exotoxin A (Pastan et al., 1995). The constructs were expressed as inclusion bodies in *E. coli* (BL21) using the pET15b vector (Novagen). Inclusion body protein was denatured and refolded essentially according to Buchner et al. (1992). The scFv-PE38 immunotoxins were then purified by sequential anion exchange chromatography on Fast Flow Q (BioRad) and Q5 resins in 20 mM Tris–HCl pH 7.4, from which they were eluted with a NaCl gradient (0–500 mM). Protein peaks were pooled according to optical density (OD) (280 nm) and SDS-Page analysis, pools were tested for activity in the MTS assay and the most potent material was used in all subsequent experiments. Purified scFv-PE38 fusion immunotoxins were monomeric by gel filtration (Sephacryl S200) with the predicted size of approximately 65 kDa molecular weight (data not shown). The diabody (scFv-(UCHT1)-PE38)₂ is a divalent scFv-PE38 fusion protein comprising the scFv of UCHT1 with a 5 amino acid VL–VH linker (Fig. 1e) compared with the 15–16 amino acid linkers used in monovalent scFv constructs (Huston et al., 1988 and Fig. 1a–d). This shorter linker promotes formation of bivalent scFv molecules by only allowing pairing of VH and VL domains from two different polypeptide chains (Holliger et al., 1993), in contrast to VH–VL pairing within the same polypeptide chain as in the monovalent scFv (Huston et al., 1988). The diabody was expressed and purified using the same methods as for scFv(UCHT1)-PE38. Sephacryl 200 gel filtration revealed that the purified diabody was a homogeneous dimeric preparation with the expected molecular weight of 130 kDa (data not shown).

DT389-scFv(UCHT1) is a recombinant fusion immunotoxin produced in *E. coli*. It comprises a diphtheria toxin fragment (DT389) fused to the N-terminus of the UCHT-1 scFv (Fig. 1b). The scFv(UCHT1)-DT389 'reverse' immunotoxin construct (Fig. 1c), with inverse domain orientation relative to the previous molecule, was assembled by cloning the UCHT1 scFv gene 5' to the DT389 fragment. These two DT389-containing constructs were assembled in the pET15b expression vector and expressed as inclusion bodies, which were refolded according to Vallera et al. (1996) and purified as described for scFv-PE38 constructs as above. The DT390-bis-scFv(UCHT1) immunotoxin (Fig. 1d) was produced by secretion from a mutant CHO cell line and purified from the supernatant as described (Liu et al., 2000). It comprises a diphtheria toxin fragment (amino acids 1–390, DT390) fused N-terminally to two tandem copies of the UCHT-1-derived scFv. The human serum albumin (HSA)-scFv(UCHT1) fusion construct was cloned into the pHILD2 vector (Invitrogen) and expressed as a secreted form in *Pichia pastoris*. HSA-scFv(UCHT1) was purified from the supernatant by ammonium sulfate precipitation, followed by cation exchange chromatography on a Bio-Rad S2 column at pH 6.0, where the protein was in the unbound fraction. The PE38 molecule, comprised the toxin domain only of *Pseudomonas* exotoxin A with an extra N-terminal lysine added, as described by Debinski and Pastan (1994). PE38 was expressed in *E. coli* with an N-terminal hexahistidine tag in the pET15b vector (Novagen), purified from the soluble fraction by Nickel affinity chromatography, and the tag removed by cleavage at the engineered thrombin site.

2.3. Competitive FACS assay for binding

Prior to competitive binding experiments, each FITC-labeled intact antibody was titrated to determine an optimal non-saturating concentration (typically 70–90% of maximal binding) for competition. All FACS incubations were in phosphate buffered saline (PBS) pH 7.2 containing 3% fetal bovine serum (FBS) and 0.1% sodium azide on ice. Binding to Jurkat human T-cells was measured for the human CD3-specific antibodies, UCHT1, BC3 and SP34, which also recognizes primate CD3 (Table 1). The murine CD3-specific antibody, 145-2C11 (Leo et al., 1987), was assayed by binding to the murine T cell line EL4. The competitive

binding protocol consisted of incubating a mixture of a dilution series (unlabeled antibody or immunotoxin) and the predetermined concentration of FITC-labeled antibody at 4 °C, for 1 h prior to adding 5×10^6 cells. After another hour at 4 °C, the cells were washed and cell-bound FITC-labeled antibody quantitated by analysis on a FACSCAN analyzer (Becton-Dickinson).

2.4. Analysis of FACS data

Binding was quantitated from FACS histograms (log FITC Fluorescence vs. cell number) generated by CELLQUEST (Becton-Dickinson) software. The software calculated the geometric mean fluorescence of

10 000 cellular events for each individual histogram. One hundred percent binding was defined as the geometric mean fluorescence (GMF) of FITC-labeled antibody in the absence of any competition, after background subtraction. In a typical experiment, the background control had a GMF of 10 and the GMF value corresponding to 100% binding was 323, after subtraction of background (e.g. for UCHT1-FITC without competition). Thus, % inhibition of binding was calculated by subtracting the GMF observed with at given competitor concentration from the 100% GMF value, observed in the absence of competition (Fig. 2). Graphical data was plotted using SIGMAPLOT or ORIGIN software. Error bars were plotted using standard error of the mean (S.E.M.), where three or more repetitions of a particular experiment were performed; otherwise typical experiments are presented. Affinity data are presented as the IC_{50} values derived from these plots as used by Burns et al. (1982) to measure the affinity of the UCHT1 antibody. IC_{50} values are determined where possible by sigmoidal curve fit analysis, otherwise estimated by x -axis intercept at 50% binding. This derivation of antibody affinity constants from IC_{50} is based on the assumptions that: (1) the system has reached equilibrium; (2) the FITC-labeled antibody binds equally well to the antigen as unlabeled Mab or immunotoxin; and (3) the thermodynamic parameters for the FITC-labeled antibody and competitor are the same. IC_{50} s were estimated by sigmoidal curve fitting, where an appropriate dose response curve could be obtained, otherwise IC_{50} s were approximated by the x -axis intercept at $y = 50\%$. Error bars are included for experiments where at least three replicates were carried out.

2.5. T-cell killing assay for immunotoxin potency determination (MTS)

The CellTiter 96® AQueous MTS (3(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium, inner salt) reagent powder was purchased from Promega (Cat. # G1111) and made up as a 2 mg/ml solution in DPBS (Dulbecco's phosphate buffered saline). Phenazine methosulfate (PMS) was purchased from Sigma (Cat. # P9625) and made up at 0.92 mg/ml in DPBS. CD3-expressing human Jurkat T-cells were grown in 75 cm² culture flasks using RPMI 1640 medium (RPMI 1640 medium supplemented with L-glutamine and 10 mM HEPES (Life Technologies, Cat. # 15630-080), 10% FBS (Life Technologies, Cat. # 16000-044), 100 U/ml penicillin, and 100 µg/ml streptomycin). They were subcultured every 2 or 3 days in fresh culture medium at the final concentration of 1 or 0.5×10^5 cells per ml, respectively. On the first day of the assay Jurkat cells were seeded into

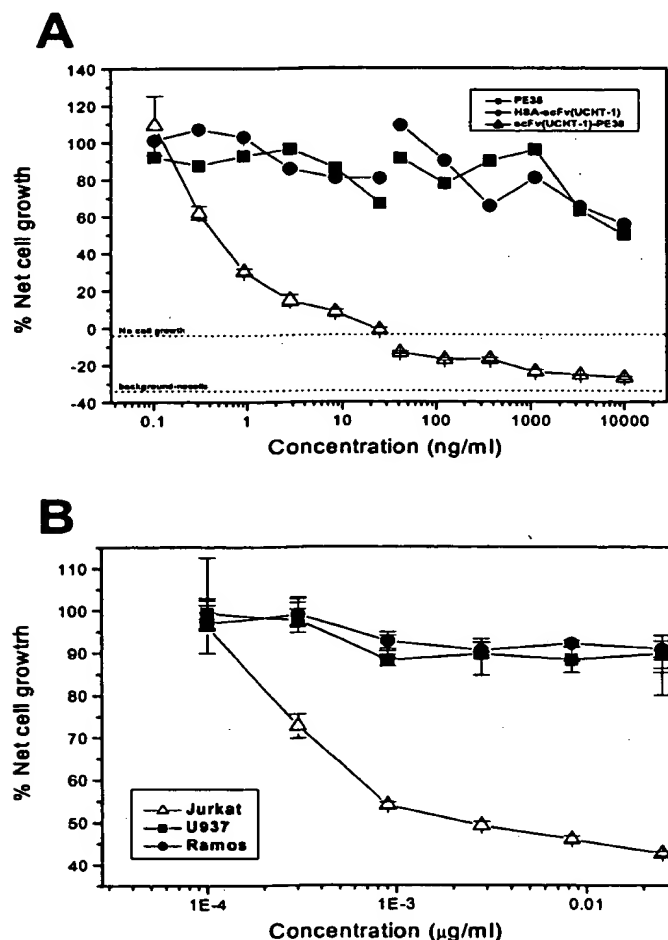


Fig. 2. Specificity of cell killing (MTS) assay. (A) Comparison of the T-cell killing ability of the scFv(UCHT1)-PE38 immunotoxin (open triangles), the PE38 toxin domain alone (filled squares) and the non-toxin containing HSA-scFv(UCHT1) fusion protein (filled circles). The break in the line plots denotes the fact that data from two independent experiments carried out across different concentration ranges were used. (B) Comparison of the cell killing ability of scFv(UCHT1)-PE38 immunotoxin on Jurkat T-cell (open triangles), Ramos B-cell (filled circles) and U937 monocytic (filled squares) lines.

Table 2
Relationship between binding affinity and cell killing potency for various anti-CD3 immunotoxins

Immunotoxin or Mab	Estimated binding affinity (M^{-1})	Cell killing IC_{50} (pM)
UCHT1 Mab	2.2×10^9	NA
BC3 Mab	1.2×10^9	NA
145-2C11 Mab	2×10^7	NA
SP34 Mab	1.5×10^7	NA
scFv(UCHT1)-PE38	1.37×10^8	14
scFv(BC3)-PE38	5×10^8	477
scFv(145-2C11)-PE38	2×10^7	7000
scFv(SP34)-PE38	1.3×10^7	58 800
DT389-scFv(UCHT1)	2.5×10^7	0.24
[scFv(UCHT1)-PE38] ₂	7.5×10^8	70
scFv(UCHT1)-DT389	1×10^8	27
DT390-biscFv(UCHT1)	9×10^9	0.031

A panel of scFv-PE38 immunotoxins derived from four different anti-CD3 monoclonal antibodies. Affinities were derived for intact Mabs or scFv immunotoxin constructs by competition with FITC-labeled appropriate parental Mabs. Potencies are shown as IC_{50} for cell killing in the MTS assay. Binding affinities were estimated from the binding IC_{50} for each construct. MTS cell killing data are indicated as IC_{50} .

96-well plates (2×10^4 cells per well) in 100 μ l of RPMI 1640 medium and cultured for 24 h at 37 °C in a humidified 5% CO_2 atmosphere.

Cell viability at the time of immunotoxin addition (day 0) was determined using the Promega CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay, as follows. One hundred microliters of culture medium were added in each well of the control plate (final volume, 200 μ l). Ten microliters of the Promega solution (obtained by mixing 1 vol of the PMS solution with 20 vol of the MTS solution) were aliquoted to each well, and cells placed at 37 °C for 4 h in a 5% CO_2 atmosphere. At the end of the incubation period, the absorbance at 490 nm was recorded using a Thermo-max ELISA plate reader (Molecular Devices). The samples of immunotoxins were prepared in culture medium by serial 3-fold dilutions. One hundred microlitres of dilution samples were aliquoted to each well, and mixed. Control wells received 100 μ l of culture medium. The plates were returned to the 5% CO_2 incubator at 37 °C. Each experimental point was performed in duplicate. The viability assay was performed as described on day 0, but without the addition of 50 μ l of culture medium, since 200 μ l is already the final volume of the medium in the wells.

To calculate IC_{50} values for a particular immunotoxin, the mean cell density (as OD 490 nm) in control untreated wells on day 4 was calculated, corrected for the day 0 reading. The values for each concentration of immunotoxin were then similarly cal-

culated and normalized using the day 4 untreated as 100% growth. The IC_{50} was derived using Microsoft EXCEL 'Forecast' function to give the immunotoxin concentration causing 50% growth inhibition.

3. Results

3.1. Specificity of the cell killing (MTS) assay

The MTS assay described above (Section 2.5) was used to determine the specific IC_{50} for T-cell killing for each immunotoxin. Using the scFv(UCHT1)-PE38 immunotoxin, the toxin domain PE38 alone and a non-toxin fusion protein HSA-scFv(UCHT1), the specificity of the assay for anti-CD3-immunotoxin-mediated cell killing using the Jurkat T-cell line was shown (Fig. 2A). The specificity of the assay for CD3 positive cells was shown in a further experiment in which the effect of the scFv(UCHT1)-PE38 immunotoxin was examined on three cell lines Jurkat (T-cell, CD3⁺), Ramos (B-cell, CD3⁻) and U937 (monocyte, CD3⁻). In this experiment, dose-dependent cell killing was only observed for the CD3⁺ cell line (Fig. 2B). Specific killing was similarly observed with two other human CD3⁺ T-cell lines Molt4 and CEM (data not shown).

3.2. Affinity and potency of immunotoxins derived from four different anti-CD3 antibodies

The initial aim of this experiment was to generate a high potency anti-CD3 scFv immunotoxin, to achieve this four antibodies, UCHT1, 145-2C11, SP34 and BC3 (Table 1), were compared in the scFv-PE38 immunotoxin format. The goal with BC3 and UCHT1 was to generate an alternative anti-human CD3 immunotoxin reagent, with SP34 the goal was to produce human-primate cross-reactive immunotoxin to aid pre-clinical development. A scFv-PE38 immunotoxin was also produced from the hamster anti-mouse CD3e-specific monoclonal antibody 145-2C11 for use in murine models of transplantation. These immunotoxins were produced and tested in the cell killing assay, where they had following rank order of potency; scFv(UCHT1)-PE38 > scFv(BC3)-PE38 > scFv(SP34)-PE38 > scFv-(145-2C11)-PE38, as detailed in Table 2. To determine the concentration of FITC-labeled antibody, at which to perform the competition experiments, a dilution series of each FITC-labeled Mab was carried out on Jurkat cells or on the EL4 murine T cell line for 145-2C11 (data not shown). Once this was determined for each FITC-labeled parent Mab, the competition assays were carried out using unlabeled Mab as control and the respective scFv-PE38 immunotoxin. UCHT1,

145-2C11 and SP34 behaved similarly as single chains in that they displayed a small loss in binding affinity (2–10 fold) when converted to the monovalent scFv-PE38 immunotoxin format, as compared with their respective Mabs (Fig. 3, panels A, C and D, respectively). In contrast, the scFv(BC3)-PE38 immunotoxin displayed significantly weaker binding to antigen (~100-fold) when compared with the BC3 Mab (Fig. 3B).

3.3. Relative affinity and efficacy of scFv(UCHT1)-PE38 and DT389-scFv(UCHT1) immunotoxins

Since the scFv derived from the UCHT1 Mab appeared to have the highest potency and binding affinity of those tested, this scFv was combined with the diphtheria toxin fragment DT389 in order to investigate if the potency could be further improved using a different toxin domain. The affinity of the UCHT1 Mab and these two monovalent immunotoxins were measured by FACS competition assay with FITC-labeled UCHT1 (Fig. 4). The affinity of the UCHT1 Mab was $2.27 \pm 0.41 \times 10^9$ per M (Fig. 4), which is in agreement with

the value obtained in studies with 125 -I labeled UCHT1 (2×10^9 per M, Burns et al., 1982). The scFv(UCHT1)-PE38 immunotoxin displayed an affinity of $1.37 \pm 0.09 \times 10^8$ per M (derived from sigmoidal curve fit analysis), approximately 10-fold lower than the parent UCHT1 antibody (Fig. 4). This loss of affinity could be explained by: (1) the transition from Mab to the single chain format; and (2) the monovalent binding of this fusion protein compared with the divalent binding of the Mab. Analysis of the affinity of the DT389-scFv(UCHT1) immunotoxin construct (2.5×10^7 per M estimated from the x -axis intercept at 50% binding, Fig. 4) revealed a 100-fold drop in CD3 binding affinity compared with the parental monoclonal antibody and a 10-fold drop compared with scFv(UCHT1)-PE38. The DT389-scFv(UCHT1) has the DT toxin domain N-terminal to the scFv domain, in contrast to scFv(UCHT1)-PE38. This C-terminal positioning of the scFv may account for the reduced binding activity of the DT389-scFv(UCHT1) molecule. In contrast to this reduced affinity, when the DT389-scFv(UCHT1) immunotoxin was tested in the cell killing assay it was found to be 60-fold more potent than the

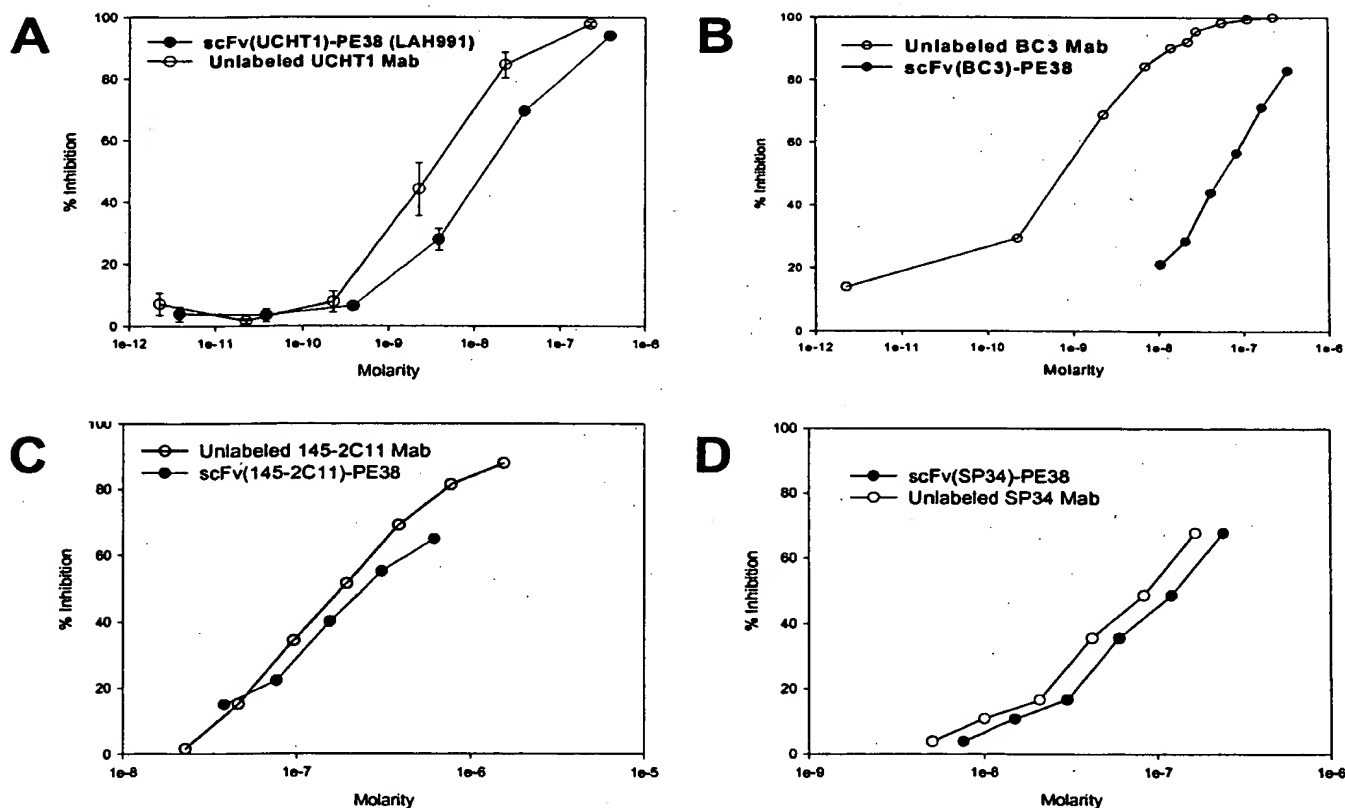


Fig. 3. Comparison of four different scFv-PE38 immunotoxins for relative affinity of antigen binding with their respective intact antibodies. Competition studies were also performed using the respective unlabeled intact antibody, shown in open circles. The scFv immunotoxins are shown in filled circles, (A) UCHT1 Mab and scFv(UCHT1)-PE38; (B) BC3 Mab and scFv(BC3)-PE38; (C) 145-2C11 Mab and scFv(145-2C11)-PE38; (D) SP34 Mab and SP34(scFv)-PE38.

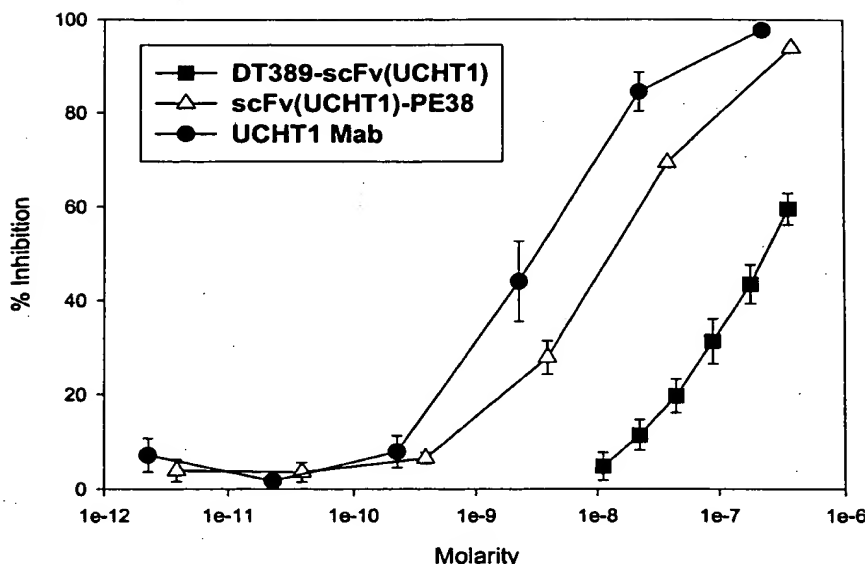


Fig. 4. The ability of DT389-scFv(UCHT1) (filled squares, $n = 7$), scFv(UCHT1)-PE38 (open triangles, $n = 9$) immunotoxins and unlabeled UCHT1 Mab (filled circles, $n = 3$) to compete FITC-labeled UCHT1 Mab binding to Jurkat T cells. Data are means of three to nine experiments as indicated, error bars show standard error of the mean.

scFv(UCHT1)-PE38 immunotoxin, Table 2). Thus, it appears that the activity of the catalytic and translocation domains of DT can more than compensate for the relatively poor CD3 binding of DT389-scFv(UCHT1).

3.4. Relative affinity and efficacy of other scFv(UCHT1)-based constructs

In order to improve the affinity, and hence, efficacy of UCHT1-scFv-based immunotoxins several additional molecules were constructed. The initial molecule was the divalent PE38-based diabody, designed with the goal of increasing the binding affinity using two scFv domains (Fig. 1). The PE38-based diabody has the same order of domains as in scFv(UCHT1)-PE38, but the short VL–VH linker peptide forces dimerization of the VH and VL domains to create a homodimer. A fusion protein of HSA with the UCHT1-scFv as a C-terminal fusion, produced in the yeast *P. pastoris*, was also tested for competitive binding. This molecule was included in the study to test the hypothesis that C-terminal positioning of the UCHT1-scFv reduces its affinity of binding, as observed with DT389-scFv(UCHT1) above.

The relative affinities of the diabody and HSA-scFv(UCHT1) molecules are shown in Fig. 5 with the UCHT1, scFv(UCHT1)-PE38 and DT389-scFv(UCHT1) from the previous experiment included for reference. It is striking that the HSA-scFv(UCHT1) molecule with the C-terminal location of the scFv, has a very similar affinity to DT389-scFv(UCHT1). Thus, in this second example, C-terminal positioning of

scFv(UCHT1) results in a loss of affinity of approximately 10-fold compared with the scFv(UCHT1)-PE38 immunotoxin. This suggests that these N-terminal domains (DT389 and HSA) similarly hinder the binding of the scFv antibody to the CD3 target antigen, possibly by steric effects.

A second PE-based UCHT1 scFv immunotoxin was generated using the 'diabody' format (Fig. 1e) to give a molecule capable of divalent antigen binding. This diabody molecule also had a reduced binding affinity (Fig. 5), approximately 5-fold lower than scFv(UCHT1)-PE38 (approximately 7.5×10^8 per M, estimated from the x intercept at 50%y). The cell killing efficacy observed ($IC_{50} = 70$ pM) was consistent with the affinity data since PE38 diabody was 5-fold less potent than scFv(UCHT1)-PE38 (Table 2). Steric hindrance, due to the presence of two toxin domains in the diabody construct may account for the observed loss of binding affinity and potency compared with monovalent scFv(UCHT1)-PE38.

In order to examine the discrepancy between the lower binding activity but higher specific cell killing activity observed with DT389-scFv(UCHT1), a construct, scFv(UCHT1)-DT389, was made. In this construct, the DT389 and scFv(UCHT1) domain order was inverted. When this construct was tested, its binding activity was improved, and was similar to that of scFv(UCHT1)-PE38 (Fig. 6). However, in the MTS cell killing assay this molecule was more than 100-fold less active than DT389-scFv(UCHT1) (IC_{50} 0.24 pM) with an IC_{50} for killing of 27 pM (Table 2). In the DT389-scFv(UCHT1) construct, the scFv is fused C-terminal

to the catalytic and translocation domains and replaces the native DT cell binding domain. The activation mechanism of the DT toxin requires internalization and cleavage of the toxin domain from the translocation domain (reviewed by Wilson and Collier, 1992). Therefore, despite the improved binding of the scFv(UCHT1)-DT389 reversed construct, the toxin activation process may be suboptimal due to the incorrect domain organization of the toxin relative to the scFv domain.

The DT390-biscFv(UCHT1) molecule is a divalent construct with tandem scFv domains located C-terminal to the DT390 toxin fragment, designed with the goal of increasing the binding affinity, and hence, potency of the immunotoxin (Liu et al., 2000). Relative affinity studies (Fig. 7) revealed that the addition of the second scFv domain increased the affinity of the molecule, resulting in a 10-fold increase of affinity compared with the monovalent DT389-scFv(UCHT1)

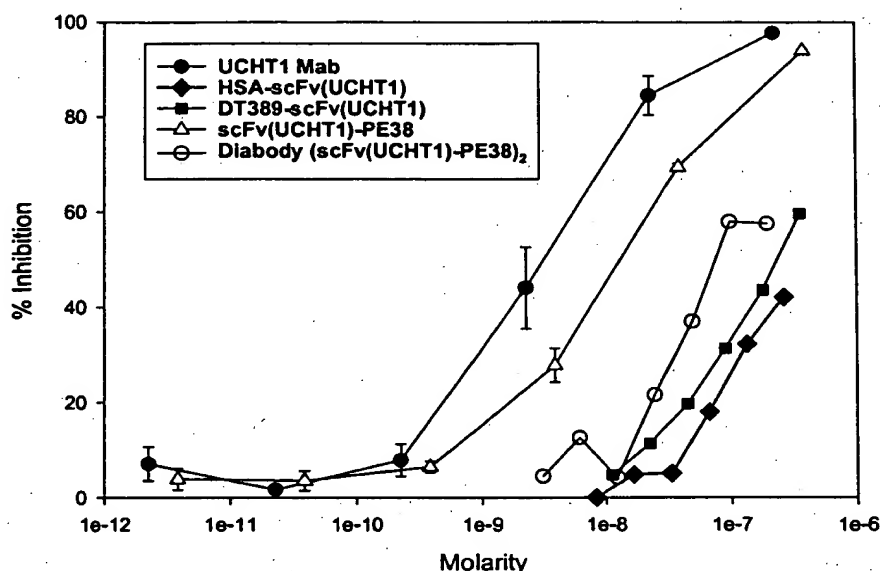


Fig. 5. Relative affinity of HSA-scFv(UCHT1) fusion protein (filled diamonds) and Diabody (scFv(UCHT1)-PE38)₂ (open circles) in competition with FITC-labeled UCHT1 binding to Jurkat T cells, typical experiments are shown. The affinity data for DT389-scFv(UCHT1) (filled squares), scFv(UCHT1)-PE38 (open triangles) and unlabeled UCHT1 Mab (filled circles) are shown for reference.

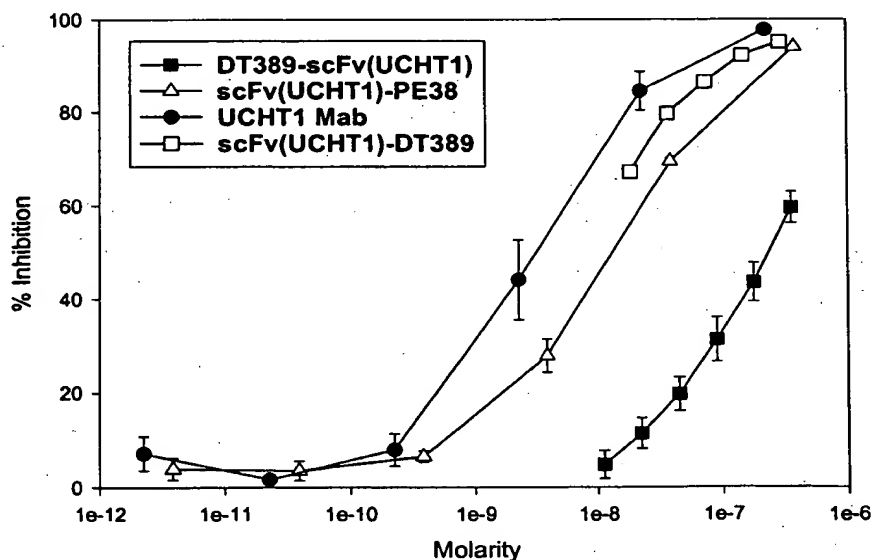


Fig. 6. Relative affinity of scFv(UCHT1)-DT389 (DT389-scFv(UCHT1) reverse construct) (open squares) in competition with FITC-labeled UCHT1 binding to Jurkat T cells. A typical experiment is shown. Affinity data for DT389-scFv(UCHT1) (filled squares), scFv(UCHT1)-PE38 (open triangles), and UCHT1 Mab (filled circles) is shown for reference.

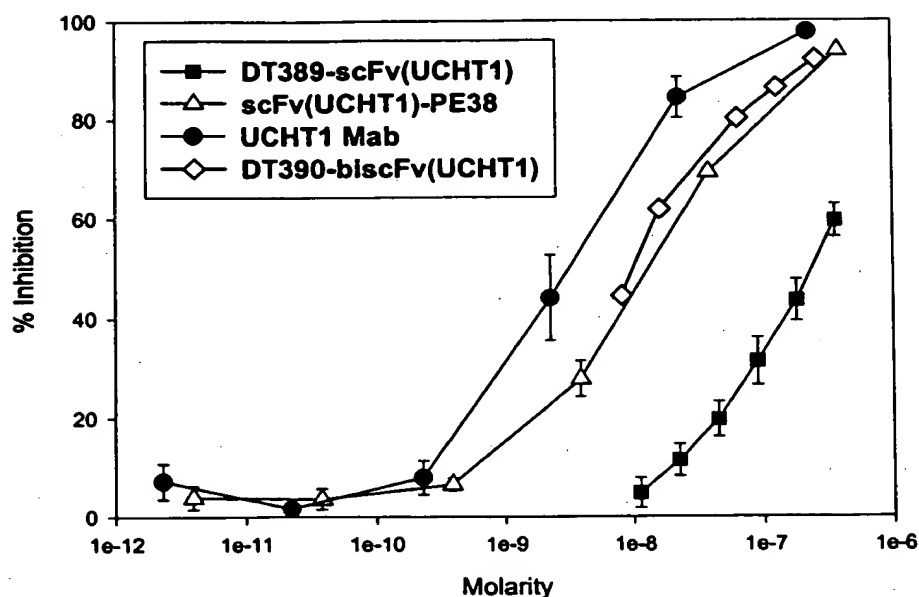


Fig. 7. Relative affinity of the DT390-bisFv(UCHT1) construct (open diamonds) in competition with FITC-labeled UCHT1 binding to Jurkat T cells, a typical experiment is shown. Affinity data for DT389-scFv(UCHT1) (filled squares), scFv(UCHT1)-PE38 (filled triangles) and unlabeled UCHT1 Mab (open circles) is shown for reference.

(Table 2). Based on a sigmoidal curve fit analysis, the affinity of DT390-bisFv(UCHT1) was approximately $9.8 \times 10^9 \text{ M}^{-1}$. This value is similar to that obtained with the monovalent scFv(UCHT1)-PE38 construct, and lower than that obtained with functionally bivalent intact UCHT1 Mab. The potency of the DT390-bisFv molecule in cell killing is approximately 10-fold higher than the monovalent DT389-scFv(UCHT1). Thus, a 10-fold increase in relative affinity appears to be exactly reflected by the 10-fold increase in potency of this divalent immunotoxin molecule, compared with the monovalent DT389-scFv(UCHT1) (Table 2).

4. Discussion

The UCHT1 antibody appears to be particularly well-suited for use in scFv immunotoxins. Not only is the antibody high affinity, it expresses well as a scFv and appears to recognize an epitope, which results in efficient internalization and subsequent activation of the toxin domain. The three anti-CD3 antibodies UCHT1, 145-2C11 and SP34, when expressed as scFv immunotoxins, all showed similar reductions in affinity (2–10-fold, compare Fig. 3A with C and D). A UCHT1 scFv with a C-terminal peptide tag displayed similar binding affinity to the scFv(UCHT1)-PE38 immunotoxin (data not shown), suggesting that the C-terminal PE38 domain does not interfere grossly with antigen binding. Such loss of affinity is consistent with the reduction from bivalent Mab binding to monova-

lent scFv binding and the fact that the scFv is a constrained engineered construct expressed in a prokaryotic system. In contrast, BC3, when expressed as a scFv-PE38 fusion, showed a larger reduction in affinity (~ 100 -fold) from that of the parent BC3 Mab (Fig. 3B). The IC_{50} s, obtained in cell killing assays, were 58.8, 7 and 0.477 nM for the 145-2C11, SP34 and BC3-based constructs, respectively. Thus, compared with the UCHT1-based scFv(UCHT1)-PE38 ($\text{IC}_{50} = 14 \text{ pM}$), scFv(145-2C11)-PE38 was 4000-fold less potent, SP34(scFv)-PE38 was 500-fold less potent and scFv(BC3)-PE38 was 40-fold less potent in the T cell killing assay, with the caveat that the 145-2C11-based molecule was assayed on the EL4 murine T cell line. These binding affinity and activity data are summarized in Table 2.

The lower potency of the other scFv constructs compared with scFv(UCHT1)-PE38 may reflect either epitope differences, resulting in less efficient processing, or differences in the absolute affinities of the antibodies. The affinities of the scFv-PE38 constructs derived from UCHT1, BC3, SP34 and 145-2C11, measured in competition with the appropriate FITC-labeled antibodies, were 1.37×10^8 , 5×10^8 , 1.3×10^7 and 2×10^7 per M, respectively (Fig. 3; Table 2). The UCHT1 and BC3 scFv-PE38 constructs thus have the highest affinities. In the same binding assay, the affinity of the UCHT1 Mab is $2.27 \pm 0.41 \times 10^9$ per M, which is in excellent agreement with the published value of 2×10^9 per M, determined by competitive binding of ^{125}I -labeled Mab

(Burns et al., 1982). In the case of BC3, the antibody was a relatively poor binder, compared with the parent Mab, when expressed as a scFv, which may explain its lower potency even though it ranks as the highest affinity scFv (Fig. 3B, Table 2). Nevertheless, the two scFv-PE38 immunotoxins (UCHT1 and BC3) that show the highest binding affinities also are the most potent. However, the rank order is reversed with scFv(BC3)-PE38 having a 4-fold higher apparent affinity than scFv(UCHT1)-PE38, while scFv(UCHT1)-PE38 is 40-fold more potent than the BC3-derived immunotoxin. The other two antibodies, SP34 and 145-2C11, when expressed as single chains, bound reasonably well to their targets, compared with their respective parent antibodies, but had lowest affinities and the lowest potencies in cell killing.

The 145-2C11 antibody was previously expressed as a scFv in a DT390-scFv(145-2C11) immunotoxin fusion molecule (Vallera et al., 1996). This molecule was able to specifically inhibit the proliferation of murine T cells in response to phytohemagglutinin or allogeneic stimulation or with an IC_{50} of 1–2 nM. Given that these molecules were assayed on mouse and human T cell lines, the observation that DT390-scFv(145-2C11) (PHA and MLR assays, Vallera et al., 1996) was 30–60-fold more potent than the scFv(145-2C11)-PE38 (MTS cell killing assay) suggests that these two molecules have activities in a similar range.

The DT-based scFv(UCHT1) immunotoxin, DT389-scFv(UCHT1) has a 10-fold lower binding affinity than the PE-based scFv(UCHT1)-PE38 immunotoxin (Fig. 4). The major structural difference between these two proteins is that in scFv(UCHT1)-PE38 the scFv is N-terminal to the toxin domain. This domain order is reversed relative to DT389-scFv(UCHT1). However, the DT389-scFv(UCHT1) immunotoxin is 60–200-fold more potent than scFv(UCHT1)-PE38 in the Jurkat cell killing assay. A similar difference was observed on other human T-cell lines such as CEM and Molt-4 (data not shown). The bivalent DT390-bisFv(UCHT1) molecule has a 10-fold higher activity in this assay than the monovalent DT389-scFv(UCHT1) immunotoxin. This difference in killing activity correlates well with the 10-fold increase in affinity demonstrated by the DT390-bisFv molecule in the competition assay (Table 2). This increased binding affinity and potency of the divalent molecule appears to result in increased in vivo potency of the DT390-bisFv(UCHT1) over the monovalent DT389-scFv(UCHT1) in a human CD3 ϵ transgenic mouse model (Thompson et al., 2001).

The N-terminally-located DT389 domain of the DT389-scFv(UCHT1) immunotoxin may cause steric inhibition of single chain binding as the affinity of this molecule for CD3 antigen is reduced. Since the DT390-bisFv binds with a similar affinity to the monovalent scFv(UCHT1)-PE38 molecule, rather than a bivalent

antibody molecule, the central scFv domain may be acting as a spacer allowing the second scFv to bind with its full affinity. The HSA-scFv fusion protein, in competition with UCHT1 Mab, displayed a curve, which was almost superimposable on that obtained with DT389-scFv(UCHT1) (Fig. 5). This suggests that any domain placed N-terminal to the scFv will reduce the binding affinity for antigen. It also somewhat validates the refolding approach for expression of UCHT1, as the eukaryotically expressed scFv has the same activity as the bacterial scFv, albeit within different fusion proteins.

The scFv(UCHT1)-DT389 molecule was produced solely to investigate whether N-terminal placement of the scFv domain would restore the affinity of the construct relative to the DT389-scFv(UCHT1), which it did. The fact that this molecule was 100-fold less potent than the conventional DT389-scFv(UCHT1) immunotoxin can be explained by the domain organization and mechanism of action of Diphtheria toxin (Wilson and Collier, 1992). The toxin domain organization dictates that the scFv in an immunotoxin be positioned to replace the toxin cell binding domain (N terminal in PE but C terminal in DT) for proper internalization, processing and activation of the toxin domain following cell binding.

The hypothesis that the presence of a protein domain fused N-terminal to an antigen binding site reduces the binding affinity is supported by affinity studies with linear F(ab')₂ (Zapata et al., 1995). This divalent molecule, comprised a tandem Fd repeat of VH-CH1-VH-CH1 with which two distinct light chains paired. Using non-binding, mutagenized variants of this molecule, the N-terminal binding Fab was shown to contribute the majority of the antigen binding energy. The C terminal Fab antigen binding site bound three to four times less well, presumably due to the steric influence of the CH1 and C κ domains immediately N-terminal to this antigen combining site.

In conclusion, there appears to be a broad correlation between affinity and potency, for different scFvs within a group of immunotoxins based on a particular toxin domain (either DT or PE). However, the DT-based immunotoxins, despite their lower binding affinity, relative to PE38-based equivalents, have a higher overall potency on the target T-cell lines studied.

Acknowledgements

Work conducted using the *P. aeruginosa* exotoxin A fragment, PE38, was performed under Commercial Evaluation Agreement L-113-9910 between Novartis Pharmaceuticals and the National Institutes of Health. Work conducted with Diphtheria toxin fusion immunotoxins was performed under a Commercial Re-

search and Development Agreement (CRADA) between Novartis Pharmaceuticals and the National Institutes of Health.

References

- Anasetti, C., Martin, P.J., Storb, R., Appelbaum, F.R., Beatty, P.G., Davis, J., Doney, K., Hill, H.F., Stewart, P., Sullivan, K.M., Witherspoon, R.P., Thomas, E.D., Hansen, J.A., 1992. Treatment of acute graft-versus-host disease with a nonmitogenic anti-CD3 monoclonal antibody. *Transplantation* 54, 844–851.
- Beverley, P.C.L., Callard, R.E., 1981. Distinctive functional characteristics of human 'T' lymphocytes defined by E resetting or a monoclonal anti-T cell antibody. *Eur. J. Immunol.* 11, 329–334.
- Buchner, J., Pastan, I., Brinkmann, U., 1992. A method for increasing the yield of properly folded recombinant fusion proteins: single-chain immunotoxins from renaturation of bacterial inclusion bodies. *Anal. Biochem.* 205, 263–270.
- Burns, G.F., Boyd, A.W., Beverley, P.C., 1982. Two monoclonal anti-human T lymphocyte antibodies have similar biologic effects and recognize the same cell surface antigen. *J. Immunol.* 129, 1451–1457.
- Clevers, H., Alarcon, B., Wileman, T., Terhorst, C., 1988. The T cell receptor/CD3 complex: a dynamic protein ensemble. *Annu. Rev. Immunol.* 6, 629–662.
- Contreras, J.L., Wang, P.X., Eckhoff, D.E., Lobashevsky, A.L., Asiedu, C., Frenette, L., Robbin, M.L., Hubbard, W.J., Cartner, S., Nadler, S., Cook, W.J., Sharff, J., Shiloach, J., Thomas, F.T., Neville, D.M. Jr, Thomas, J.M., 1998. Peritransplant tolerance induction with anti-CD3 immunotoxin: a matter of proinflammatory cytokine control. *Transplantation* 65, 1159–1169.
- Debinski, W., Pastan, I., 1994. An immunotoxin with increased activity and homogeneity produced by reducing the number of lysine residues in recombinant *Pseudomonas* exotoxin. *Bioconj. Chem.* 5, 40–46.
- Duvic, M., Cather, J., Maize, J., Frankel, A.E., 1998. DAB₃₈₉IL2 diphtheria fusion toxin produces clinical responses in tumor stage cutaneous lymphoma. *Am. J. Hematol.* 58, 87–90.
- Foss, F.M., Saleh, M.N., Krueger, J.G., Nichols, J.C., Murphy, J.R., 1998. Diphtheria toxin fusion proteins. *Curr. Top. Microbiol. Immunol.* 234, 63–81.
- Frankel, A.E., Tagge, E.P., Willingham, M.C., 1995. Clinical trials of targeted toxins. *Semin. Cancer Biol.* 6, 307–317.
- Gilliland, L.K., Norris, N.A., Marquart, H., Tsu, T.T., Hayden, M.S., Neubauer, M.G., Yelton, D.E., Mittler, R.S., Ledbetter, J.A., 1996. Rapid and reliable cloning of antibody variable regions and generation of recombinant single chain antibody fragments. *Tissue Antigens* 47, 1–20.
- Holliger, P., Prospero, T., Winter, G., 1993. 'Diabodies': small bivalent and bispecific antibody fragments. *Proc. Natl. Acad. Sci. USA* 90, 6444–6448.
- Huston, J.S., Levinson, D., Mudgett-Hunter, M., Tai, M.S., Novotny, J., Margolies, M.N., Ridge, R.J., Brucoleri, R.E., Haber, E., Crea, R., 1988. Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 85, 5879–5883.
- Knechtle, S.J., Vargo, D., Fechner, J., Zhai, Y., Wang, J., Hanaway, M.J., Scharff, J., Hu, H., Knapp, L., Watkins, D., Neville, D.M. Jr, 1997. FN18-CRM9 immunotoxin promotes tolerance in primate renal allografts. *Transplantation* 63, 1–6.
- LeMaistre, C.F., Saleh, M.N., Kuzel, T.M., Foss, F., Platanius, L.C., Schwartz, G., Ratain, M., Rook, A., Freytes, C.O., Craig, F., Reuben, J., Nichols, J.C., 1998. Phase I trial of a ligand fusion protein DAB₃₈₉IL2 in lymphomas expressing the receptor for IL-2. *Blood* 91, 399–405.
- Leo, O., Foo, M., Sachs, D.H., Samelson, L.E., Bluestone, J.A., 1987. Identification of a monoclonal antibody specific for a murine CD3 polypeptide. *Proc. Natl. Acad. Sci. USA* 84, 1374–1378.
- Liu, Y.Y., Gordienko, I., Mathias, A., Ma, S., Thompson, J., Woo, J.-H., Neville, D.M. Jr, 2000. Expression of an anti-CD3 single-chain immunotoxin in a mutant CHO cell line. *Protein Exp. Purif.* 19, 304–311.
- Matsudaira, P., 1987. Sequence from picomol quantities of proteins electroblotted onto polyvinylidene fluoride membranes. *J. Biol. Chem.* 262, 10035–10038.
- Nooij, F.J., Jonker, M., Balner, H., 1986. Differentiation antigens on rhesus monkey lymphocytes. II. Characterization of RhT3, a CD3-like antigen on T cells. *Eur. J. Immunol.* 16, 981–984.
- Pai, L.H., Pastan, I., 1998. Clinical trials with *Pseudomonas* exotoxin immunotoxins. *Curr. Top. Microbiol. Immunol.* 234, 83–96.
- Pastan, I.H., Pai, L.H., Brinkmann, U., Fitzgerald, D.J., 1995. Recombinant toxins: new therapeutic agents for cancer. *Ann. NY Acad. Sci.* 758, 345–354.
- Saleh, M.N., LeMaistre, C.F., Kuzel, T.M., Foss, F., Platanius, L.C., Schwartz, G., Ratain, M., Rook, A., Freytes, C.O., Craig, F., Reuben, J., Sams, M.W., Nichols, J.C., 1998. Anti-tumor activity of DAB₃₈₉IL2 fusion toxin in mycosis fungoides. *J. Am. Acad. Dermatol.* 39, 63–73.
- Sambrook, J., Fritsch, E., Maniatis, T., 1989. *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor laboratory Press, NY, USA.
- Sancho, J., Ledbetter, J.A., Choi, M.S., Kanner, S.B., Deans, J.P., Terhorst, C., 1992. CD3-zeta surface expression is required for CD4-p56lck-mediated upregulation of T cell antigen receptor-CD3 signaling in T cells. *J. Biol. Chem.* 267, 7871–7879.
- Shalaby, M.R., Shepard, H.M., Presta, L., Rodrigues, M.L., Beverley, P.C., Feldmann, M., Carter, P., 1992. Development of humanized bispecific antibodies reactive with cytotoxic lymphocytes and tumor cells overexpressing the HER2 protooncogene. *J. Exp. Med.* 175, 217–225.
- Thomas, J.M., Neville, D.M. Jr, Contreras, J.L., Eckhoff, D.E., Meng, G., Lobashevsky, A.L., Wang, P.X., Huang, Z.Q., Verbanac, K.M., Haisch, C.E., Thomas, F.T., 1997. Preclinical studies of allograft tolerance in rhesus monkeys: a novel anti-CD3 immunotoxin given peritransplant with donor bone marrow induces operational tolerance to kidney allografts. *Transplantation* 64, 124–135.
- Thomas, F.T., Ricordi, C., Contreras, J.L., Hubbard, W.J., Jiang, X.L., Eckhoff, D.E., Cartner, S., Bilbao, G., Neville, D.M., Thomas, J.M., 1999. Reversal of naturally occurring diabetes in primates by unmodified islet xenografts without chronic immunosuppression. *Transplantation* 27, 846–854.
- Thomas, J.M., Eckhoff, D.E., Contreras, J.L., Lobashevsky, A.L., Hubbard, W.J., Moore, J.K., Cook, W.J., Thomas, F.T., Neville, D.M. Jr, 2000. Durable donor-specific T and B cell tolerance in rhesus macaques induced with peritransplantation anti-CD3 immunotoxin and deoxyspergualin: absence of chronic allograft nephropathy. *Transplantation* 69, 2497–2503.
- Thompson, J., Stavrou, S., Weetall, M., Hexham, J.M., Digan, M.E., Wang, Z., Woo, J.H., Yu, Y., Mathias, A., Liu, Y.Y., Ma, S., Gordienko, I., Neville, D.M. 2001. Improved binding

- of a bivalent single chain immunotoxin results in increased toxicity for in vivo T-cell depletion, *Protein Eng.*, in press.
- Vallera, D.A., Panoskaltsis-Mortari, A., Jost, C., Ramakrishnan, S., Eide, C.R., Kreitman, R.J., Nicholls, P.J., Pennell, C., Blazar, B.R., 1996. Anti-graft versus host disease effect of DT390-anti-CD3sFv, a single chain immunotoxin specifically targeting the CD3 ϵ moiety of the T-cell receptor. *Blood* 88, 2342–2353.
- Wilson, B.A., Collier, R.J., 1992. Diphtheria toxin and *Pseudomonas* exotoxin A: active site structure and enzymic mechanism. *Curr. Top. Microbiol. Immunol.* 175, 27–41.
- Zapata, G., Ridgway, J.B., Mordenti, J., Osaka, G., Wong, W.L., Bennett, G.L., Carter, P., 1995. Engineering linear F(ab')₂ fragments for efficient production in *E. coli* and enhanced antiproliferative activity. *Protein Eng.* 10, 1057–1062.

Single-Chain Immunotoxins Directed at the Human Transferrin Receptor Containing *Pseudomonas* Exotoxin A or Diphtheria Toxin: Anti-TFR(Fv)-PE40 and DT388-Anti-TFR(Fv)

JANENDRA K. BATRA, DAVID J. FITZGERALD, VIJAY K. CHAUDHARY, AND IRA PASTAN*

Laboratory of Molecular Biology, Division of Cancer Biology, Diagnosis and Centers,
National Cancer Institute, Bethesda, Maryland 20892

Received 5 November 1990/Accepted 18 January 1991

Two single-chain immunotoxins directed at the human transferrin receptor have been constructed by using polymerase chain reaction-based methods. Anti-TFR(Fv)-PE40 is encoded by a gene fusion between the DNA sequence encoding the antigen-binding portion (Fv) of a monoclonal antibody directed at the human transferrin receptor and that encoding a 40,000-molecular-weight fragment of *Pseudomonas* exotoxin (PE40). The other fusion protein, DT388-anti-TFR(Fv), is encoded by a gene fusion between the DNA encoding a truncated form of diphtheria toxin and that encoding the antigen-binding portion of antibody to human transferrin receptor. These gene fusions were expressed in *Escherichia coli*, and fusion proteins were purified by conventional chromatography techniques to near homogeneity. In anti-TFR(Fv)-PE40, the antigen-binding portion is placed at the amino terminus of the toxin, while in DT388-anti-TFR(Fv), it is at the carboxyl end of the toxin. Both these single-chain immunotoxins kill cells bearing the human transferrin receptors. However, anti-TFR(Fv)-PE40 was usually more active than DT388-anti-TFR(Fv), and in some cases it was several-hundred-fold more active. Anti-TFR(Fv)-PE40 was also more active on cell lines than a conjugate made by chemically coupling the native antibody to PE40, and in some cases it was more than 100-fold more active.

Immunotoxins made by chemically coupling tumor-specific monoclonal antibodies to bacterial or plant toxins, including *Pseudomonas* exotoxin (PE) and diphtheria toxin (DT) have been shown to possess in vitro and in vivo antitumor activity (10, 19, 24). Both PE and DT kill cells by ADP-ribosylating elongation factor 2, thereby inhibiting protein synthesis. X-ray crystallographic and mutational analyses show the PE molecule to be made up of three distinct domains, namely, an amino-terminal cell-binding domain, a middle translocation domain, and a carboxyl-terminal activity domain (1, 12). DT, which is divided into two chains, A and B, linked by a disulfide bridge, is arranged functionally in the orientation opposite to that of PE. Chain B of DT, which is at the carboxyl end, is responsible for receptor binding, and chain A, at the amino end, contains the enzymatic activity (22, 23). Apparently the last 150 amino acids of DT are responsible for its cell-binding activity (21-23).

We have constructed immunotoxins by attaching antibodies either to native PE that has an M_r of 66 kDa or to truncated forms of PE that lack its cell-binding domain and have an M_r of about 40 kDa (referred to as PE40). Immunotoxins made with PE40 or LysPE40, a PE40 derivative with an extra lysine residue at its amino terminus that facilitates coupling to antibodies, have greater specificity for target cells because PE cannot bind to its cellular receptor (3, 13, 20). One such immunotoxin, anti-TFR-LysPE40, was shown to be highly cytotoxic to cells expressing the human trans-

ferrin receptor. When given intraperitoneally to mice, it caused regression of A431 tumors growing as subcutaneous xenografts (3). Immunoconjugates with low nonspecific toxicity have also been made by using modified DT with mutations in its cell-binding domain (17, 25).

Immunotoxins constructed by chemical cross-linking are heterogeneous and are difficult to produce in large amounts because the antibody and toxin must be made and purified separately and then conjugated in a reaction that often has a low yield. To address this problem, we have made two recombinant single-chain immunotoxins directed at the interleukin-2 receptor. The two are termed anti-Tac(Fv)-PE40 and DT388-anti-Tac(Fv). The Fv fragment is the smallest binding unit of an antibody which consists of a light- and a heavy-chain variable domain. Both single-chain immunotoxins consist of the variable domains of a monoclonal antibody directed at the interleukin-2 receptor arranged in a single-chain form and linked to the amino terminus of PE40 (2, 9) or to the carboxyl end of a truncated form of DT (7). Single-chain immunotoxins are constructed by starting with hybridoma RNA, using polymerase chain reaction (PCR) to amplify the DNA encoding the variable heavy and variable light chains of the antibodies, and inserting this DNA into an expression vector containing PE40 sequences (4). We used this rapid cloning technique and now report the construction of two single-chain immunotoxins, anti-TFR(Fv)-PE40 and DT388-anti-TFR(Fv). In anti-TFR(Fv)-PE40, the single-chain anti-TFR(Fv), containing the variable heavy chain of a monoclonal antibody (HB21) to the human transferrin receptor (anti-TFR) (11) linked through a peptide linker to its variable light chain, is fused to the amino terminus of PE40. In DT388-anti-TFR(Fv), the single-chain anti-TFR(Fv) is linked to the carboxyl end of the truncated DT. Both of these single-chain

FIG. 1. (A) S₁ X, XbaI; S, SalI; (C) Expression p

immunotoxins v
fied to near ho
anti-TFR(Fv) w
man transferri
differences in th
were detected.

Plasmid const
expression plas
TFR(Fv)-PE40

* Corresponding author.

and *Bam*HI. Several positive clones were checked for protein expression in BL21 (λ DE3).

Figure 1C shows the plasmid used for the expression of DT388-anti-TFR(Fv). To construct this plasmid, PCR with pJB-anti-TFR(Fv)-1108 as template was used to amplify DNA for anti-TFR(Fv) such that it had *Nde*I at the 5' end and *Eco*RI at the 3' end. The PCR-amplified fragment was restricted with *Nde*I and *Eco*RI and ligated to pVC-DT-IL2 (6, 7) digested with *Nde*I and *Eco*RI. The recombinants were screened with *Hind*III, *Bam*HI, and *Nde*I. pVC-DT1-IL2 contains the first 388 codons of DT with the initiator methionine fused to cDNA encoding human interleukin-2 (6).

Protein expression and purification. BL21 (λ DE3) cells were transformed with the plasmids pJB anti-TFR(Fv)-1108 or pJB DT388-anti-TFR(Fv). The cells were grown in superbroth containing 100 μ g of ampicillin per ml at 37°C. At an A_{650} of 0.8, cells were induced with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG). Cells were harvested 90 min later, and the location of the fusion protein was determined as described elsewhere (5). The fusion protein was isolated from the inclusion bodies by denaturation in 7 M guanidine HCl and renaturation by rapid dilution in phosphate-buffered saline. After dialysis, the renatured protein was applied to an 8-ml Q-Sepharose column. Proteins were batch eluted from the column with 0.1, 0.35, and 1 M NaCl. The pool containing the desired fusion protein (0.35 M salt eluate) was diluted and loaded onto a Mono Q 10/10 fast protein liquid chromatography column. The fusion protein was eluted by a linear gradient of 0 to 0.5 M NaCl in 0.02 M Tris (pH 7.4). Further purification was achieved by gel filtration chromatography on a TSK 250 column.

Cytotoxicity assay. Cytotoxic activities of anti-TFR(Fv)-PE40, anti-TFR-LysPE40, and DT388-anti-TFR(Fv) were determined by assaying the inhibition of protein synthesis as measured by [3 H]leucine incorporation into total cell protein (9). The results are expressed as percentage of control, to which no toxin was added. For competition experiments, 20 μ g of antibody was added per well prior to the addition of the toxin (9).

Binding studies. 125 I-labeled HB21 (20 μ Ci/ μ g) was added as a tracer at 1.5 ng per assay with various concentrations of competitor and 4×10^5 HUT102 cells or 5×10^5 A431 cells in 0.2 ml of binding buffer (RPMI 1640 with 10% fetal bovine serum, 100 μ g of human immunoglobulin per ml, and 0.1% sodium azide) and incubated at room temperature with shaking for 2 h. At the end of the incubation, cells were washed three times with the binding buffer and counted in a gamma counter (9).

Other methods. Anti-TFR-LysPE40 was constructed as described elsewhere (3). Protein was assayed by Bradford's method with Bio-Rad protein assay reagent (3a). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was done by the method of Laemmli (14).

RESULTS

Plasmid construction, protein expression, and purification. The structure of a plasmid encoding anti-TFR(Fv)-PE40 is shown in Fig. 1A. The assembled gene is under the control of a bacteriophage T7 promoter. The plasmid expresses the first ~110 amino acids of anti-TFR heavy chain, a 15-amino-acid linker containing (Gly₄Ser)₃, the first ~100 amino acids of anti-TFR light chain, and amino acids 253 to 613 of PE. The expression plasmid pJB-DT388-anti-TFR(Fv) shown in Fig. 1C encodes the first 388 amino acids of DT fused to the variable heavy domain of anti-TFR antibody, which is

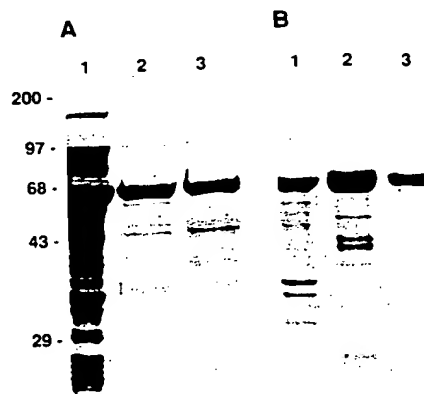


FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of anti-TFR(Fv)-PE40 (A) and DT388-anti-TFR(Fv) (B) at different steps of purification. Lane 1, Inclusion bodies; lane 2, Mono Q pool; lane 3, TSK gel filtration pool. Gel was stained with Coomassie blue. Numbers at left are molecular sizes in kilodaltons.

connected to its variable light chain through the (Gly₄Ser)₃ linker. Anti-TFR(Fv)-PE40 and DT388-anti-TFR(Fv) were expressed in *E. coli* (BL21 λ DE3), and after induction with IPTG, the fusion proteins were contained primarily in the inclusion bodies. The fusion protein from the inclusion bodies was denatured in 7 M guanidine HCl and renatured by rapidly dilution in phosphate-buffered saline. Purification was performed by chromatography on a Q-Sepharose anion-exchange column and then on a Mono Q fast protein liquid chromatography column. The pool containing the active protein was finally purified by gel filtration on a TSK 250 column. The purity of anti-TFR(Fv)-PE40 and DT388-anti-TFR(Fv) at each purification step is shown in Fig. 2A and B, respectively. The protein after gel filtration chromatography appeared to be >90% pure and was used for all subsequent studies.

Cytotoxicity and specificity of anti-TFR(Fv)-PE40. As shown in Fig. 3, anti-TFR(Fv)-PE40 was extremely active and inhibited the protein synthesis of A431 cells in a dose-dependent manner with a 50% inhibitory dosage (ID_{50}) of 0.02 ng/ml, which is equivalent to 0.2 pM (Table 1). The cytotoxic effect was blocked by competition with excess

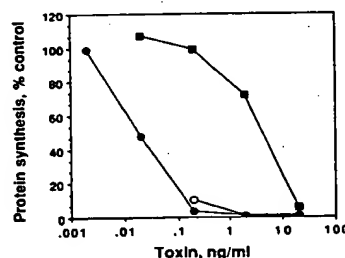


FIG. 3. Cytotoxicity of anti-TFR(Fv)-PE40 on A431 cells. Cells were incubated with the toxin for 16 to 20 h and pulsed with [3 H]leucine, and radioactivity was measured in the trichloroacetic acid-precipitable protein. Results are shown as percentage of control, to which no toxin was added. Symbols: ●, anti-TFR(Fv)-PE40 alone; ○, anti-TFR(Fv)-PE40 plus 20 μ g of OVB3; ■, anti-TFR(Fv)-PE40 plus 20 μ g of HB21. HB21 is a monoclonal antibody to the human-transferrin receptor produced by the hybridoma used as the source of RNA for cloning the variable domains.

TABLE 1

Cell line	
A431	Epi
KB	Epi
MCF7	Bre
OVCAR3	Ov
HUT102	Ad
HT29	Co
PC3	Pro
LNCAP	Pro
DU145	Pro
L929	Fil
Swiss 3T3	Fil

* Ratio of act on the same cell

anti-TFR, w the cytotox TFR(Fv)-PE fusion prote synthesis in reflecting th

Comparis toxin and cl PE40 was compared conjugate c shown in synthesis i ranging be anti-TFR-I ID_{50} s were single-chai mouse cell ferrin rece

Binding To compa that of th were perf these stu compete cells was

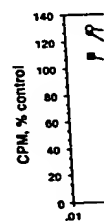


FIG. 4. HB21, v anti-TFR(Fv)-PE40 (○) labeled ti

TABLE 1. Activity of anti-TFR(Fv)-PE40 on various human cell lines

Cell line	Origin	ID ₅₀ (pM)		Relative activity ^a
		Anti-TFR(Fv)-PE40	Anti-TFR-LysPE40	
A431	Epidermoid carcinoma	0.18	20	111
KB	Epidermoid carcinoma	0.10	75	750
MCF7	Breast carcinoma	0.14	13.5	96
OVCAR3	Ovarian carcinoma	8.0	1,000	125
HUT102	Adult T-cell leukemia	37.0	50	1.3
HT29	Colon carcinoma	45.0	160	3
PC3	Prostate carcinoma	58.5	280	5
LNCAP	Prostate carcinoma	2.6	10	4
DU145	Prostate carcinoma	92.0	400	4
L929	Fibroblast (mouse)	>30,000	>10,000	
Swiss 3T3	Fibroblast (mouse)	>30,000	>10,000	

^a Ratio of activity of anti-TFR(Fv)-PE40 to activity of anti-TFR-LysPE40 on the same cell line.

anti-TFR, whereas OVB3, a control antibody, did not block the cytotoxicity, demonstrating the specificity of anti-TFR(Fv)-PE40 (Fig. 2) for the transferrin receptor. Also, the fusion protein at 2 µg/ml (30 nM) did not inhibit protein synthesis in either mouse Swiss 3T3 or mouse L929 cells, reflecting the species specificity of the antibody (Table 1).

Comparison of cytotoxic activities of recombinant immunotoxin and chemical conjugate. The activity of anti-TFR(Fv)-PE40 was assayed on a variety of human cell lines and compared with that of anti-TFR-LysPE40, the chemical conjugate composed of the native antibody and PE40 (3). As shown in Table 1, anti-TFR(Fv)-PE40 inhibited protein synthesis in all the human cell lines studied, with ID₅₀s ranging between 0.10 and 92 pM. The chemical conjugate anti-TFR-LysPE40 was also toxic to these cells. However, ID₅₀s were up to 750-fold lower than that of the recombinant single-chain immunotoxin. Both molecules were inactive on mouse cells, demonstrating their specificity for human transferrin receptor (Table 1).

Binding of anti-TFR(Fv)-PE40 to the transferrin receptor. To compare the binding activity of anti-TFR(Fv)-PE40 with that of the native antibody, competition binding analyses were performed with HUT102 and A431 cells (Fig. 4). In these studies, the abilities of various chimeric toxins to compete for the binding of ¹²⁵I-HB21 (anti-TFR) to A431 cells was determined. Anti-TFR(Fv)-PE40 was shown to

TABLE 2. Activity of DT388-anti-TFR(Fv) on various human cell lines

Cell line	Toxin ID ₅₀ (pM)	Ratio of DT-anti-TFR(Fv) to anti-TFR(Fv)-PE40 ^a
A431	88	0.002
KB	82	0.001
MCF7	59	0.002
OVCAR3	34	0.2
HUT102	10	3.7
HT29	16	2.8
L929	15,000	

^a Ratio of ID₅₀s of DT-anti-TFR(Fv) to those of anti-TFR(Fv)-PE40 on the same cell line.

compete for binding to the human transferrin receptor very efficiently; its binding affinity was similar to that of HB21 on A431 cells and approximately twofold lower on HUT102 cells. In the same experiments, the chemical conjugate anti-TFR-LysPE40 was tested and found to compete much less well than native HB21. The binding affinity of anti-TFR-LysPE40 appears to be about 10-fold lower than that of the native anti-TFR antibody (Fig. 4).

Cytotoxicity and specificity of DT388-anti-TFR(Fv). DT388-anti-TFR(Fv) was also prepared, tested, and found to be cytotoxic to several human cell lines, with ID₅₀s ranging between 10 and 100 pM (Table 2). Its cytotoxic activity was also blocked by excess antibody, demonstrating the specificity of DT388-anti-TFR(Fv) for the transferrin receptor (data not shown). Furthermore, DT388-anti-TFR(Fv) was inactive on mouse L929 cells, demonstrating its specificity for human cells (Table 2). When compared with anti-TFR(Fv)-PE40, the DT immunotoxin was 400- to 800-fold less active on A431, KB, and MCF7 cells but 2- to 3-fold more active on HUT102 and HT29 cells.

DISCUSSION

We have constructed two new single-chain immunotoxins directed at the human transferrin receptor. One of these is anti-TFR(Fv)-PE40, which consists of the variable regions of HB21, a monoclonal antibody to the human transferrin receptor, joined to PE40, a truncated form of PE devoid of its binding domain. This fusion protein was expressed in *E. coli* and purified to near homogeneity. Anti-TFR(Fv)-PE40 was found to be very cytotoxic to cells bearing human transferrin receptors. The cytotoxicity was specific, as shown by competition with excess native antibody. To demonstrate that it should be possible to make single-chain immunotoxins that have the same antigen-binding domain but a different toxin and to compare their activities on different cell types, we constructed a second single-chain immunotoxin, DT388-anti-TFR(Fv), containing a truncated form of DT lacking DT binding activity. This fusion protein also was found to be cytotoxic to cells expressing the human transferrin receptor. Unexpectedly, large differences in the activities of the two single-chain immunotoxins were observed. On some cell lines (A431, KB, and MCF7), anti-TFR(Fv)-PE40 was at least 100-fold more active than DT388-anti-TFR(Fv). On two cell lines (HUT102 and HT29), DT388-anti-TFR(Fv) was about threefold more active. However, in no case was DT388-anti-TFR(Fv) much more active than the PE-containing immunotoxin, whereas the reverse was observed. The basis of these differences in activities remains to be explored. The mechanism by which immuno-

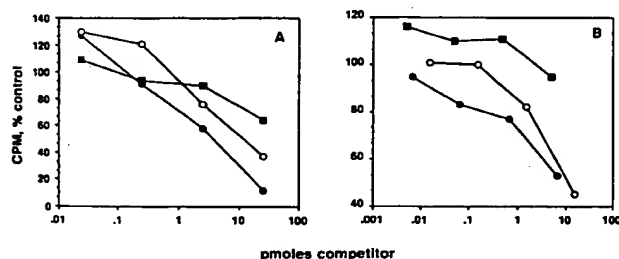


FIG. 4. Competition binding analysis of anti-TFR antibody, HB21, versus anti-TFR(Fv)-PE40. Competition of anti-TFR(Fv)-PE40 (○), HB21-LysPE40 (■), and native HB21 (●) with ¹²⁵I-labeled tracer HB21 on HUT102 (A) and A431 (B) cells.

toxins kill cells is complex and involves binding to the receptor, internalization via coated pits into endocytic vesicles, and proteolytic processing of the toxin to a form which can be translocated into the cytosol. It seems unlikely to us that the difference in the activities is related to binding or the rate of internalization; instead, the difference probably arises during the proteolytic processing or translocation steps.

Recently, we have shown that PE is processed by a cellular protease to produce an N-terminal 28-kDa and a C-terminal 37-kDa fragment. Only the 37-kDa fragment is translocated to the cytosol (18). The proteolytic clip occurs at or near Arg-279 in domain I of PE. Production of the 37-kDa fragment appears to be essential for toxicity, since mutant PE molecules PEgly²⁷⁶, PEgly²⁷⁹, and PEhis^{274,276,279} that do not produce this fragment are unable to kill target cells (18). The amino acid sequence at the carboxyl end of PE is REDLK, and changing it to REDL or KEDLK does not affect the cytotoxic activity of the toxin (8). Mutant PE molecules ending with KDEL or RDEL were fully active, whereas LDER at the carboxyl end resulted in an inactive molecule, even though the mutant PE molecule ending with LDER was processed normally, generating the 28- and 37-kDa fragments (18). This observation suggests that the sequence at the carboxyl end of PE acts as a recognition sequence to assist translocation of the toxin from an endocytic compartment to the cytosol (8). Thus, at least two specific recognition events occur within the cells, one within domain II that leads to processing and one at the carboxyl end of domain III. Olsnes and colleagues, using a system in which whole cells are exposed to a low-pH environment, have presented strong evidence that a fragment of DT closely resembling the entire A chain is the final processed form that reaches the cytosol (15). DT contains a trypsin-sensitive region with three closely spaced arginines at positions 190, 192, and 193. Cleavage of the toxin to produce A and B fragments appears to occur in a stochastic manner after either of these arginine residues (16). The site of cleavage appears to be one of the factors affecting translocation, since only A fragments lacking both Arg-192 and Arg-193 were found to be translocation competent (16). DT does not contain a terminal sequence resembling REDLK, indicating that it probably has a mechanism of cellular entry different from that of PE. From the present study, it appears that despite identical ADP ribosylation activities, both PE and DT are probably processed or translocated or both by different mechanisms, resulting in the differential sensitivity of the cells towards these toxins.

Competition studies showed the binding affinity of anti-TFR(Fv)-PE40 to be very similar to that of the native bivalent antibody on A431 cells and HUT102 cells. Since anti-TFR(Fv)-PE40 is monovalent, this was a somewhat surprising result. Previously, we had found that anti-Tac(Fv)-PE40 bound threefold less well than anti-Tac to the human interleukin 2 receptor-bearing cells, HUT102. On comparing the cytotoxic activity of anti-TFR(Fv)-PE40 with that of a chemical conjugate, anti-TFR-Lys(PE40), the recombinant protein was found to be much more active on all the human tumor cells investigated. This difference was in part explained by the diminished binding of the chemical conjugate to the transferrin receptor, probably as a result of treatment of the antibody with the modifying 2-iminothiolane during the preparation of the immunotoxin (3). However, other steps in immunotoxin action must be differentially affected as well.

One objective of creating chimeric toxins is their potential usefulness as antitumor agents. Our previous study shows

that anti-TFR-LysPE40 has a very potent in vivo antitumor activity against A431 epidermoid carcinomas. Because anti-TFR(Fv)-PE40 is very cytotoxic to A431 and KB cells in culture (Table 1), we plan to test anti-TFR(Fv)-PE40 in animals with these tumors to determine the in vivo antitumor activity of this single-chain immunotoxin.

In summary, we have shown in the present study as well as in our earlier report (20) that the single-chain immunotoxins made by the fusion of antigen-binding and toxin domains can retain the binding affinity of the native antibody and are generally more active than the chemical conjugates. Also, active single-chain immunotoxins can be made with different toxin moieties, thus placing antigen-binding portions either at the amino terminus or the carboxyl terminus as required. With this information in hand, it should be possible to make active single-chain immunotoxins from the wide variety of toxins (plant, bacterial, and animal) that are now being made by chemical coupling methods (10, 19, 24).

ACKNOWLEDGMENTS

We thank J. Evans and A. Gaddis for typing the manuscript and S. Neal for the photography.

REFERENCES

1. Allured, V. S., R. J. Collier, S. F. Carroll, and D. B. McKay. 1986. Structure of exotoxin A of *Pseudomonas aeruginosa* at 3.0 Å resolution. *Proc. Natl. Acad. Sci. USA* 83:1320-1324.
2. Batra, J. K., D. FitzGerald, M. Gately, V. K. Chaudhary, and I. Pastan. 1990. Anti-Tac(Fv)-PE40, a single chain antibody *Pseudomonas* exotoxin fusion protein directed at the interleukin-2 receptor bearing cells. *J. Biol. Chem.* 265:15198-15202.
3. Batra, J. K., Y. Jinno, V. K. Chaudhary, T. Kondo, M. C. Willingham, D. J. FitzGerald, and I. Pastan. 1989. Antitumor activity in mice of an immunotoxin made with anti-transferrin receptor and a recombinant form of *Pseudomonas* exotoxin. *Proc. Natl. Acad. Sci. USA* 86:8545-8549.
- 3a. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
4. Chaudhary, V. K., J. K. Batra, M. G. Gallo, M. C. Willingham, D. J. FitzGerald, and I. Pastan. 1990. A rapid method of cloning functional variable region antibody genes in *E. coli* as single chain immunotoxins. *Proc. Natl. Acad. Sci. USA* 87:1066-1070.
5. Chaudhary, V. K., D. J. FitzGerald, S. Adhya, and I. Pastan. 1987. Activity of a recombinant fusion protein between transforming growth factor alpha and *Pseudomonas* toxin. *Proc. Natl. Acad. Sci. USA* 84:4538-4542.
6. Chaudhary, V. K., D. J. FitzGerald, and I. Pastan. Submitted for publication.
7. Chaudhary, V. K., M. G. Gallo, D. J. FitzGerald, and I. Pastan. A recombinant single-chain immunotoxin composed of anti-Tac variable regions and a truncated diphtheria toxin. *Proc. Natl. Acad. Sci. USA* 87:9491-9494.
8. Chaudhary, V. K., Y. Jinno, D. FitzGerald, and I. Pastan. 1990. *Pseudomonas* exotoxin contains a specific sequence at the carboxyl terminus that is required for cytotoxicity. *Proc. Natl. Acad. Sci. USA* 87:308-312.
9. Chaudhary, V. K., C. Queen, R. P. Junghans, T. A. Waldmann, D. J. FitzGerald, and I. Pastan. 1989. A recombinant immunotoxin consisting of two antibody variable domains fused to *Pseudomonas* exotoxin. *Nature (London)* 339:394-397.
10. FitzGerald, D., and I. Pastan. 1989. Targeted toxin-therapy for the treatment of cancer. *J. Natl. Cancer Inst.* 81:1455-1463.
11. Haynes, B. F., M. Hemlar, T. Cotner, D. L. Mann, G. S. Eisenbarth, J. L. Strominger, and A. S. Fauci. 1981. Characterization of a monoclonal antibody (5E9) that defines a human cell surface antigen of cell activation. *J. Immunol.* 127:347-351.
12. Hwang, J., D. J. P. FitzGerald, S. Adhya, and I. Pastan. 1987.

Func
deletio
136.
13. Kondo,
Pastan.
ified p
domain
14. Laemm
assemb
227:680
15. Moska
induce
Biol. C
16. Moska
Transl
Biol. C
17. Murph
fusion:
opmer
Kluwe
18. Ogata
1990.
ase re
is tran

- Functional domains of pseudomonas exotoxin identified by deletion analysis of the gene expressed in *E. coli*. *Cell* 48:129-136.
13. Kondo, T., D. FitzGerald, V. Chaudhary, S. Adhya, and I. Pastan. 1988. Activity of immunotoxins constructed with modified pseudomonas exotoxin A lacking the cell recognition domain. *J. Biol. Chem.* 263:9470-9475.
 14. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227:680-685.
 15. Moskaug, J. O., K. Sandvig, and S. Olsnes. 1988. Low pH induced release of diphtheria toxin A-fragment in Vero cells. *J. Biol. Chem.* 263:2518-2525.
 16. Moskaug, J. O., K. Sletten, K. Sandvig, and S. Olsnes. 1989. Translocation of diphtheria toxin A-fragment to the cytosol. *J. Biol. Chem.* 264:15709-15713.
 17. Murphy, J. R. 1988. Diphtheria related peptide hormone gene fusions: a molecular genetic approach to chimeric toxin development, p. 123-140. *In* A. E. Frankel (ed.), *Immunotoxins*. Kluwer Academic Publishers, Boston.
 18. Ogata, M., V. K. Chaudhary, I. Pastan, and D. J. FitzGerald. 1990. Processing of *Pseudomonas* exotoxin by a cellular protease results in the generation of a 37,000-Da toxin fragment that is translocated to the cytosol. *J. Biol. Chem.* 265:20678-20685.
 19. Pastan, I., M. C. Willingham, and D. J. P. FitzGerald. 1986. Immunotoxins. *Cell* 47:641-648.
 20. Pirker, R., D. J. P. FitzGerald, T. C. Hamilton, R. F. Ozols, M. C. Willingham, and I. Pastan. 1985. Anti-transferrin-receptor antibody linked to *Pseudomonas* exotoxin as a model immunotoxin in human ovarian carcinoma cell lines. *Cancer Res.* 45:751-757.
 21. Rolf, J. M., H. M. Gaudin, and L. Eidels. 1990. Localization of the diphtheria toxin receptor-binding domain to the carboxyl terminal Mr approximately 6000 region of the toxin. *J. Biol. Chem.* 265:7331-7337.
 22. Uchida, T., A. M. Pappenheimer, Jr., and R. Greary. 1973. Diphtheria toxin and related proteins. I. Isolation and properties of mutant proteins serologically related to diphtheria toxin. *J. Biol. Chem.* 248:3838-3844.
 23. Uchida, T., A. M. Pappenheimer, Jr., and A. A. Harper. 1972. Reconstitution of diphtheria toxin from two non-toxic cross reacting mutant proteins. *Science* 175:901-903.
 24. Vitetta, E. S., R. J. Fulton, R. D. May, M. Till, and J. W. Uhr. 1987. Redesigning nature's poisons to create anti-tumor reagents. *Science* 238:1098-1104.
 25. Youle, R. J., L. Greenfield and V. G. Johnson. 1988. Genetic engineering of immunotoxins, p. 113-122. *In* A. E. Frankel (ed.), *Immunotoxins*. Kluwer Academic Publishers, Boston.